

**THROMBOSPONDIN FRAGMENTS AND USES THEREOF
IN CLINICAL ASSAYS FOR CANCER AND GENERATION
OF ANTIBODIES AND OTHER BINDING AGENTS**

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CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. provisional application 60/405,494 filed August 23, 2002; and U.S. application serial No. 10/419,462, filed April 21, 2003.

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FIELD OF THE INVENTION

The present invention relates to assays for blood levels of one or more thrombospondin fragments as a diagnostic test for cancers and other diseases, the use of such fragments and/or derivatives thereof to generate specific antibodies and other binding agents and/or to use as calibrators, competitors, and/or indicators in an assay, and to the fragments 15 themselves.

BACKGROUND OF THE INVENTION

Thrombospondin (TSP), also known as TSP-1, is a multimeric glycoprotein comprised of identical monomers. The monomers migrate at an apparent molecular weight 20 of approximately 185 kDa in SDS-polyacrylamide electrophoretic gels under reducing conditions. The predominant multimer is a trimer, which migrates at an apparent molecular weight of approximately 450 kDa on non-reducing gels. The molecular weights by sedimentation equilibrium are similar, at 135 kDa for monomers and 420 kDa for trimers. The predicted molecular weight from just the sequence of amino acyl residues in the 25 monomer is 127,524 Da, which does not include contributions from glycosylation and β -hydroxylation. The thrombospondin glycoprotein is produced by platelets and is released upon platelet activation from platelet α -granules, along with many other proteins, such as platelet-derived growth factor, β -thromboglobulin, fibronectin, fibrinogen, and platelet factor-4 (see Chapter 1, "An introduction to the thrombospondins" in The Thrombospondin Gene Family by JC Adams, RP Tucker, & J Lawler, Springer-Verlag: New York, 1995, pp. 1-9, but especially p. 2; and Chapter 3, "The secondary and tertiary structure of the thrombospondins," *ibidem* pp. 43-56, especially Table 3.1). Thrombospondin is known to be involved in biological processes such as cell adhesion, proliferation and chemotaxis. It has also been reported that thrombospondin may be involved in the progression of malignant

tumors. Furthermore, thrombospondin has been reported to be highly expressed in many human malignant tissues and in surrounding stroma and/or endothelium and has been reported to be present in higher than normal levels in the plasma of cancer patients. (e.g., Qian and Tuszyński, *Proc. Soc. Exp. Biol. Med.*, 212:199-207, 1996; de Fraipont F et al. *Trends Mol. Med.*, 7:401-407, 2001).

5 Despite the foregoing, as for any potential diagnostic test, it would be desirable to increase the specificity and sensitivity of such tests. To that end, the present inventor has discovered that thrombospondin is present in the blood and blood plasma in relatively small amounts compared to fragments of thrombospondin, and this finding is true in the blood and 10 blood plasma of cancer patients as well. This discovery provided a basis for the present inventions related to novel diagnostic assays that are more specific, more sensitive, more easily calibrated, and in some cases distinguish these thrombospondin fragments from each other and from thrombospondin itself.

15 **BRIEF SUMMARY OF THE INVENTION**

Important aspects of the invention are diagnostic methods and related kits that are based on the detection and quantification of thrombospondin fragments and/or thrombospondin in bodily fluids, especially plasma. Foremost among those diagnostic methods are those that detect or monitor the status of a cancer.

20 Aspects of the invention closely related to the diagnostic methods are thrombospondin fragments that are detected in the plasma, thrombospondin fragments that can be used to induce an antibody of interest for use in a diagnostic method or can be used in a competition-type or non-competitive diagnostic assay.

25 **Thrombospondin fragments of the invention**

In one aspect, the invention is a purified thrombospondin fragment that has been extracted from a bodily fluid, especially plasma, said fragment being one within a molecular weight range selected from the group consisting of 80 to 110 kDa, 40 to 60 kDa, and 20 to 35 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide 30 bond reduction. Their uses include, but are not limited to, a) the induction of an antibody of interest, b) induction of an antibody for a diagnostic method, c) use in a competition-type diagnostic assay, d) as a reference molecule in an assay for a thrombospondin fragment or fragments or thrombospondin of human subjects, and e) the immunization of an animal. In a

closely related aspect, the invention is a polypeptide or modified polypeptide, made by recombinant and/or chemical techniques, that has the identical primary structure as one of said purified thrombospondin fragments or a portion thereof. Such chemical techniques include, but are not limited to, glycosylation, β -hydroxylation, alkylation and reduction.

5 In particular embodiments, the fragment's molecular weight is one within a molecular weight range selected from the group consisting of 80 to 95 kDa, 47 to 53 kDa, and 27 to 33 kDa. Specific examples of fragment molecule weights are 85, 90, 50, and 30 kDa. Preferably, the fragment is one found in human plasma.

10 In a related aspect, the invention is a purified and/or synthetic thrombospondin fragment or portion thereof, said fragment being one that starts between amino acid I-165 (just after the N12/I peptide) and V-263 (the start of the procollagen homology domain), inclusive (*i.e.*, inclusive of I-165 and V-263), and ends between amino acid K-412 (the end of the reported collagen type V-binding region) and I-530 (the end of the domain of type 1 repeats), inclusive. Preferred are such fragments that start at between N-230 and G-253, 15 inclusive (at or near the start of the domain of interchain disulfide bonds, I-241, which is the first residue downstream [meaning towards the C-terminus of the full protein] of a predicted cleavage site for chymotrypsin and/or a chymotrypsin-like protease), and end at between V-400 and S-428, inclusive (at or near a predicted chymotrypsin cleavage site, F-414, that falls two residues after the end of the collagen type V-binding region), said portion being at least 20 3 amino acyl acids in length (preferably at least 4 amino acyl residues in length, more preferably at least 6 amino acyl residues).

25 In a further related aspect, the invention is a purified and/or synthetic thrombospondin fragment or portion thereof, said fragment being one that starts between amino acid I-165 (just after the N12/I peptide) and V-263 (the start of the procollagen homology domain), inclusive, and ends between amino acid I-530 (the end of the type 1 repeats) and R-733 (the end of the first type 3 repeat), inclusive. Preferably such a fragment starts between N-230 and G-253, inclusive, and ends between D-527 and S-551, inclusive, which is at or near a predicted chymotrypsin cleavage site, F-539, in the first type 2 repeat; 30 said portion being at least 3 amino acyl acids in length (preferably at least 4 amino acyl residues in length, more preferably at least 6 amino acyl residues).

In a still further related aspect, the invention is a purified and/or synthetic thrombospondin fragment or portion thereof, said fragment being one that starts between

amino acid I-165 (just after the N12/I peptide) and V-263 (the start of the procollagen homology domain), inclusive, and ends between amino acid R-792 (the end of the third type 3 repeat) and Y-982 (the third of the predicted chymotrypsin cleavage sites in the C-terminal domain), inclusive. Preferably such a fragment starts between N-230 and G-253, inclusive, 5 and ends between G-787 and V-811, inclusive, which is at or near a predicted chymotrypsin cleavage site, Y-799, in the fourth type 3 repeat; said portion being at least 3 amino acyl acids in length (preferably at least 4 amino acyl residues in length, more preferably at least 6 amino acyl residues). Protein molecular weights here were computed using standard computational aids (such aids are available, for example, at the web site of the 10 Bioinformatics Organization, Inc., http://bioinformatics.org/sms/prot_mw.html; see Stothard, P. 2000. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *BioTechniques* 28: 1102-1104) and adjusted upwards to account for post-translational modifications. Predicted cleavage sites for chymotrypsin (and 15 any closely related protease) were identified using tools available from the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) (See <http://us.expasy.org/cgi-bin/peptidecutter/peptidecutter.pl>) and were limited to predicted sites of at least 80% probability. The uses of said fragments and portions include, but are not limited to, the induction and/or screening of an antibody and/or another binding 20 agent of interest in a diagnostic method and use in a diagnostic assay. In particular embodiments, the invention is one of the specified fragments, rather than a portion thereof. In additional embodiments, a fragment and/or a portion can incorporate or be linked to a label and/or a carrier.

Throughout, wherever reference is made to a fragment or a portion thereof (or an immunoreactive portion thereof), it is understood that the fragment is a preferred 25 embodiment of the invention. It is also understood throughout this Application that immunogenic portions, immunoreactive portions, and/or epitopes are generally six amino acyl residues long or longer, but an occasional portion or epitope can be shorter. Such shorter portions or epitopes are also contemplated.

Five additional aspects are:

30 1) A purified and/or synthetic thrombospondin fragment, said fragment being at least 6 contiguous amino acyl residues in length, and wherein the fragment comprises a protease-resistant core domain or a part thereof, said domain or part thereof being selected from the group consisting of a domain of inter-chain disulfide bonds, an oligomerization domain, a

procollagen-like domain, a type 1 repeat, a type 2 repeat, and a type 3 repeat, said part being at least 6 amino acyl residues in length.

2) A purified and/or synthetic thrombospondin fragment, said fragment being at least 6 contiguous amino acyl residues in length, and wherein the fragment comprises an amino acid sequence selected from the group consisting of TEENKE (SEQ ID NO:1), CLQDSIRKVTEENKE (which includes an N-terminal Cys added to aid conjugation) (SEQ ID NO:2), LQDSIRKVTEENKE (SEQ ID NO:3), EGEARE (SEQ ID NO:4), PQMNGKPCEGEARE (SEQ ID NO:5), EDTDLD (SEQ ID NO:6), YAGNGIICGEDTDLD (SEQ ID NO:7), CNSPSPQMNGKPCEGEAR (SEQ ID NO:8), 10 RKVTEENKELANELRRP (SEQ ID NO:9), CRKVTEENKELANELRRP (which includes an N-terminal Cys added to aid conjugation) (SEQ ID NO:10), PQMNGKPCEGEAR (SEQ ID NO:11), CEGEAR (SEQ ID NO:12), and RKVTEENKE (SEQ ID NO:13). (In particular embodiments the fragment comprises two, or even all of the foregoing sequences).

3) a purified and/or synthetic thrombospondin fragment, said fragment being at least 15 6 contiguous amino acyl residues in length, and wherein the fragment comprises a collagen type V binding domain or a portion thereof.

4) A purified and/or synthetic thrombospondin fragment, said fragment being at least 6 contiguous amino acyl residues in length, and wherein the fragment comprises an epitope for binding at least one of the following commercially available antibodies, each of which 20 recognizes a ~450kDa (non-reduced) protein that is specifically identified as thrombospondin (the TSP Ab numbering, e.g., "TSP Ab-2", comes from Lab Vision Corporation, Fremont, CA, which currently has a web site at <http://www.labvision.com/> ; clone designations refer to the hybridoma clone that produces a particular monoclonal antibody) It is also understood that said fragment includes a fragment that can be designed 25 to bind a pre-existing monoclonal antibody, through the use of peptide scanning analysis, competition experiments, and other methods known in the art (for an example of such methods, see Corada M et al. Monoclonal antibodies directed to different regions of vascular endothelial cadherin extracellular domain affect adhesion and clustering of the protein and modulate endothelial permeability. *Blood*. 2001 Mar 15;97(6):1679-84). It is also 30 understood that the current invention includes, but is not limited to, uses of pre-existing antibodies independent of a purified and/or synthetic fragment, some of which uses are also listed below.

TSP Ab-2 (Clone D4.6): This antibody is stated to react against reduced and non-reduced protein, and its epitope is in the calcium-binding domain of TSP (C-terminal 50-kDa piece of the 120-kDa fragment from protease digestion of Ca-replete TSP). The calcium-binding region is generally considered to be in the type 3 repeats (TSP residues 698-925). For 5 example, it is expected that TSP Ab-2 will bind thrombospondin but not the 30-kDa circulating fragment. This antibody can be used to detect and/or quantify TSP and/or a circulating fragment; distinguish thrombospondin from a circulating fragment; and/or distinguish one or more fragments from each other. It shows no cross-reaction with fibronectin, fibrinogen, and von Willebrand factor. Its binding to thrombospondin is 10 enhanced by EDTA i.e. at low [Ca²⁺].

TSP Ab-4 (Clone A6.1): This antibody is stated to react against reduced and non-reduced protein, and its epitope is in the collagen type V-binding domain. This antibody binds thrombospondin, and the applicant has discovered that it binds the three major TSP 15 fragments in human plasma. Thus, this antibody can be used to detect and/or quantify TSP and/or a circulating fragment or fragments. In combination with another antibody or binding agent, it can be used in an assay to distinguish thrombospondin from a circulating fragment; and/or to distinguish one or more fragments from each other. As an example meant to be 20 illustrative and not restrictive, TSP Ab-4 is used to capture TSP and circulating fragments, and then the other antibody or binding agent is used for detection, but is able to distinguish TSP from a fragment or fragments, or one fragment from another. It is understood that TSP Ab-4 also binds thrombospondin and thrombospondin fragments from important non-human sources as well, including but not limited to the dog. Thus, the use of this antibody and/or a 25 similar binding agent in an assay for a thrombospondin fragment or fragments in a sample from a non-human source, such as dog, is contemplated. This antibody shows no cross-reaction with fibronectin, fibrinogen, and von Willebrand factor. This antibody inhibits thrombospondin-collagen interaction, and its binding to thrombospondin is unaffected by glycosaminoglycans (e.g. hyaluronic acid, chondroitin sulfate, and heparin). Also, its binding is enhanced by EDTA i.e. at low conc. of Ca²⁺.

30 TSP Ab-5 (Clone B5.2): This antibody is stated to react against reduced and non-reduced protein, and its epitope is in a 10-kDa fragment present at the junction of type 2 and type 3 repeats. The junctional region is listed elsewhere as residues 674-697, but this is only 24

residues and less than 10-kDa, so the epitope is less precisely mapped. It is expected that this antibody will bind TSP but not the 30-kDa circulating fragment. Thus, this antibody can be used to detect and/or quantify TSP and/or a circulating fragment or fragments; distinguish thrombospondin from a circulating fragment; and/or distinguish one or more fragments from 5 each other. It shows no cross-reaction with fibronectin, fibrinogen, and von Willebrand factor.

TSP Ab-9 (Clone MBC 200.1): This antibody is stated to react against reduced and non-reduced protein, and its epitope is in the N-terminal heparin-binding domain of 10 thrombospondin. Thus, it should bind to thrombospondin but not to major circulating fragments. In Western blotting, Ab-9 reacts with a 25kDa peptide (heparin-binding domain) from thermolysin digests of thrombospondin that is not disulfide bonded to any other region of the thrombospondin molecule. Heparin efficiently inhibits the binding of Ab-9 to 15 thrombospondin. Thus, this antibody can be used to detect and/or quantify TSP; and/or distinguish thrombospondin from a circulating fragment or fragments. This antibody is not suitable for detecting all major fragments in the circulation.

TSP Ab-8 (rabbit polyclonal antibody): Recognizes a ~450kDa (non-reduced) or 180kDa (reduced) protein, identified as TSP. This antibody, which is a rabbit polyclonal, can be 20 used in sandwich ELISAs for capture or detection and in competitive ELISAs. The applicant has discovered that it binds the three major TSP fragments in human plasma. Thus, this antibody can be used to detect and/or quantify TSP and/or a circulating fragment or fragments. In combination with another antibody or binding agent, it can be used in an assay to distinguish thrombospondin from a circulating fragment; and/or to distinguish one 25 or more fragments from each other.

As an example meant to be illustrative and not restrictive, one takes the difference between (a) the result of an assay using an antibody or binding agent that binds TSP and the major circulating fragments in plasma, *versus* (b) the result of an assay using an antibody or binding agent that binds TSP but not major fragments. The antibody or binding agent in (a) is selected from the group consisting of TSP Ab-4, TSP Ab-8, TSP Ab-11, and an antibody 30 or binding agent that binds TSP and the major circulating fragments in plasma. The antibody or binding agent in (b) is selected from the group consisting of TSP Ab-3, TSP Ab-6, TSP Ab-9, and an antibody or binding agent that binds TSP but none of the major

circulating fragments. Said assay in (a) detects TSP plus fragments; said assay in (b) detects TSP; said difference, (a) minus (b), thereby gives a quantification of fragments without TSP. Likewise, differences can be taken between (c) the result of an assay using an antibody or binding agent that binds TSP and a subset of the major circulating fragments in plasma,
5 *versus* the result of (a), above, to obtain a quantification of the fragment or fragments not detected in (c). Differences can also be taken of the result of (c) *versus* (b), above, to obtain a quantification of the fragment or fragments detected in (c) but without the signal from TSP. The antibody or binding agent in (c) is selected from the group consisting of TSP Ab-2, TSP Ab-5, TSP Ab-1, TSP Ab-7, and an antibody or binding agent that binds TSP and
10 only a subset of the major circulating fragments.

TSP Ab-11 (Clones D4.6 + A6.1 + MBC 200.1): The Ab-11 cocktail is designed for sensitive detection of thrombospondin by Western blotting. This antibody cocktail shows no cross-reaction with fibronectin, fibrinogen, and von Willebrand factor. Because it is a
15 mixture of TSP Ab-2, TSP Ab-4, and TSP Ab-9, it detects TSP and the three major TSP fragments in human plasma. Thus, this antibody can be used to detect and/or quantify TSP and/or a circulating fragment or fragments. In combination with another antibody or binding agent, it can be used in an assay to distinguish thrombospondin from a circulating fragment; and/or to distinguish one or more fragments from each other. It can also be used in an assay
20 for TSP and/or a TSP fragment or fragments in a sample from a non-human source, such as a dog.

Other antibodies that are useful, even though they have been disclosed only as binding non-reduced protein include, but are not limited to TSP Ab-1, TSP Ab-3, TSP Ab-6,
25 and TSP Ab-7, which are described in more detail immediately below:

TSP Ab-1 (Clone A4.1): This antibody is stated to bind the N-terminal half of the central stalk-like region of thrombospondin. This region is recovered as a 50kDa fragment after
30 chymotryptic digestion of thrombospondin. Thus, Ab-1 may be used to detect and/or quantify TSP and/or a circulating fragment or fragments; distinguish thrombospondin from a circulating fragment or fragments; and/or distinguish one or more fragments from each other. TSP Ab-1 shows no cross-reaction with fibronectin, fibrinogen, and von Willebrand

factor. It inhibits the adhesion of human melanoma G361 cells, keratinocytes, squamous carcinoma cells, and rat smooth muscle cells to thrombospondin. It does not inhibit aggregation of thrombin-induced platelets. This antibody is stated to block the anti-angiogenic activity of thrombospondin by inhibiting its binding to TSP-Receptor/CD36.

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TSP Ab-3 (Clone C6.7): This antibody is stated to bind the platelet or cell-binding domain at the extreme C-terminus of TSP and should therefore distinguish TSP from fragments. Thus, this antibody can be used to detect and/or quantify TSP; and/or distinguish thrombospondin from a circulating fragment or fragments. This antibody is not suitable for 10 detecting the three major fragments in the circulation. Heparin or EDTA may marginally affect binding of Ab-3 to thrombospondin. Ab-3 blocks thrombospondin-mediated agglutination of fixed red blood cells. It shows no effect on thrombospondin-mediated agglutination of fixed, activated platelets. It inhibits both thrombin- and A23187-induced aggregation of washed, live (not fixed) platelets without affecting the secretion of serotonin. 15 Ab-3 inhibits adhesion of melanoma G361 cells to thrombospondin, and blocks the binding of C-terminal domain to Integrin-Associated Protein (IAP)/CD47.

TSP Ab-6 (Clone A2.5): This antibody has been shown to immunoprecipitate thrombospondin. This antibody shows no cross-reaction with fibronectin, fibrinogen, and 20 von Willebrand factor. Its epitope localizes in the heparin-binding domain of thrombospondin, and therefore, heparin efficiently inhibits the binding of Ab-6 to thrombospondin. Thus, this antibody can be used to detect and/or quantify TSP; and/or distinguish thrombospondin from a circulating fragment or fragments. This antibody is not suitable for detecting the three major fragments in the circulation. Hyaluronic acid and 25 chondroitin sulfate show no inhibition at low concentration and only partially inhibit over the concentration range at which heparin abolishes the binding. Thrombospondin binds with high affinity to a sulfated glycolipid or sulfatide found on red cell and platelet membranes. Ab-6 blocks the binding of thrombospondin to sulfatides at low concentrations. Ab-6 immunoprecipitates a 25kDa peptide (heparin-binding domain) from chymotryptic digests of 30 thrombospondin that is not disulfide bonded to any other region of the thrombospondin molecule. This antibody inhibits the hemagglutination of trypsinized, glutaraldehyde-fixed human erythrocytes by purified thrombospondin. It also inhibits the agglutination of fixed, activated platelets by thrombospondin. It does not inhibit either thrombin- or A23187-

induced aggregation of washed, live platelets. Ab-6 does not bind to reduced and alkylated thrombospondin or thrombospondin transferred to nitrocellulose membrane after SDS-PAGE.

5 **TSP Ab-7 (Clone HB8432):** This antibody is stated to bind type 2 repeats. Thus, Ab-7 may be used to detect and/or quantify TSP and/or a circulating fragment or fragments; distinguish thrombospondin from a circulating fragment or fragments; and/or distinguish one or more fragments from each other. It shows no cross-reaction with fibronectin or any other serum or platelet proteins except thrombospondin. Its epitope localizes in the EGF-like repeats
10 (type 2) in the stalk region of human thrombospondin (disulfide-bonded core remaining after trypsin digestion).

All of the antibodies listed above can be purchased from Lab Vision Corporation, Fremont, CA currently with a web site at <http://www.labvision.com/>. See also the published
15 literature such as, for TSP Ab-4, Galvin NJ et al. Interaction of human thrombospondin with types I-V collagen: direct binding and electron microscopy. *J Cell Biol.* 1987 May;104(5):1413-22). It is also understood that alternative antibodies may also be generated against any of the abovementioned epitopes.

5) A purified and/or synthetic thrombospondin fragment, said fragment being at least
20 6 contiguous amino acyl residues in length, and wherein the fragment does not comprise at least one fibrinogen-binding region selected from the group consisting of (1) a fibrinogen-binding domain within a 210-kDa fragment of TSP, where said 210-kDa fragment is composed of three 70-kDa fragments that contain the region of interchain disulfide bonds, the procollagen homology region, and the type 1 and type 2 repeats, (2) a fibrinogen-binding
25 region in the amino-terminal domain of thrombospondin, (3) a fibrinogen-binding region in an 18-kDa amino-terminal heparin-binding domain of thrombospondin, and (4) a region corresponding to synthetic peptide N12/I encompassing amino acid residues 151-164 (I-151 to P-164) of the N-terminal domain of thrombospondin-1. In a particular embodiment, the fragment does not comprise any of the fibrinogen-binding regions in the group.

30 For each of the 5 additional aspects, the molecular weight of the thrombospondin fragment does not exceed 110 kDa; alternatively does not exceed 55 kDa; or alternatively does not exceed 35 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction. The fragments of the 5 additional aspects of the invention can

be used to induce antibodies (and/or other binding molecules) of interest in the diagnostic methods or can be used in diagnostic assays, for example, as calibrators, indicators, and/or competitors. It is understood that a fragment can be derivatized, for example, to incorporate and/or be coupled to a label and/or a carrier.

5 A fragment that can be as little as 6 amino acyl residues in length is preferably immunoreactive. A typical method for immunizations comprises coupling the peptide to a carrier, such as keyhole limpet hemocyanin or ovalbumin. Said couplings to a carrier are also contemplated in the current invention.

10 The inclusion of the central protease-resistant core domain in the definition of the fragments follows from considerations discussed elsewhere herein. This domain is considered to comprise locations in the mature thrombospondin protein selected from the group consisting of: a domain of interchain disulfide bonds (around Cys-252 and Cys-256, preferably residues 241-262); the procollagen homology domain (residues 263-360); the type 1 repeats (residues 361-530); the type 2 repeats (residues 531-673); there is a short 15 segment (residues 674-697) between the type 2 repeat domain and the type 3 repeat domain; and then the type 3 repeats (residues 698-925); see Figure 1 of this Application for examples of protease-resistant fragments that have been reported after artificial digestions *in vitro*; Chapter 2, "The primary structure of the thrombospondins" in The Thrombospondin Gene Family by JC Adams, RP Tucker, & J Lawler, Springer-Verlag: New York, 1995, pp. 11-42, particularly p. 12; and Chapter 6, "Mechanistic and functional aspects of the interactions of thrombospondins with cell surfaces," *ibidem*, pp. 105-157, particularly p. 115. Interchain disulfide bonds (in the region of residues 241-262) are often preserved in protease-resistant fragments. The term "mature", as used here to refer to the mature thrombospondin protein sequence, means without the 18- to 22-residue signal peptide sequence, here assumed to be 20 18 residues, following The Thrombospondin Gene Family by JC Adams *et al.* 1995; see the full human thrombospondin sequence given below in this text; see also Figure 1 of this application, and the discussions thereof. Nevertheless, it is understood that GenBank file NM_003246.1, also listed as GI:4507484, currently identifies nucleotide residues "112..204" 25 as encoding the signal peptide, which implies a signal peptide of 31 amino acyl residues).

30 The identification of these peptides, TEENKE (SEQ ID NO:1), LQDSIRKVTEENKE (SEQ ID NO:3), EGEARE (SEQ ID NO:4), PQMNGKPCEGEARE (SEQ ID NO:5), EDTDLD (SEQ ID NO:6), YAGNGIICGEDTDLD (SEQ ID NO:7), CNSPSPQMNGKPCEGEAR (SEQ ID NO:8), RKVTEENKELANELRRP (SEQ ID NO:9),

PQMNGKPCEGEAR (SEQ ID NO:11), CEGEAR (SEQ ID NO:12), and RKVTEENKE (SEQ ID NO:13) was achieved by computerized surveys of thrombospondin, the surveys done by request at commercial sources to identify immunogenic regions (epitopes), but these surveys identified many peptides with immunogenic regions, and so the surveys were 5 followed by selection of relevant peptides and/or epitopes based on knowledge of circulating thrombospondin fragments. Other peptides and/or epitopes listed in this application were similarly identified.

A criterion that a fragment comprises an immunogenic and/or immunoreactive portion from a collagen type V binding domain follows from the published properties (e.g., 10 Galvin NJ et al. Interaction of human thrombospondin with types I-V collagen: direct binding and electron microscopy. *J Cell Biol.* 1987 May;104(5):1413-22) of the commercially available TSP Ab-4 antibody used below to detect thrombospondin fragments of interest in the plasma.

The collagen V-binding domain of thrombospondin has been mapped to the amino 15 acid sequence corresponding to the region between valine(333) and lysine(412) (V-333 to K-412, using the single-letter symbols V and K for their respective amino acids), inclusive, of human thrombospondin-1 (Takagi T et al. A single chain 19-kDa fragment from bovine thrombospondin binds to type V collagen and heparin. *J Biol Chem* 268:15544-15549, 1993; as mentioned above, numbers here refer to the mature thrombospondin protein, that is, 20 without the 18- to 22-residue signal peptide sequence, here assumed to be 18 residues). This region would include a portion of the procollagen homology region of thrombospondin and all or nearly all of the first type 1 repeat of thrombospondin (see Chapter 2, "The primary structure of the thrombospondins" in The Thrombospondin Gene Family by JC Adams, RP Tucker, & J Lawler, Springer-Verlag: New York, 1995, pp. 11-42, but especially p. 24).

25 The criterion that the fragment comprise an epitope for binding the commercially available TSP Ab-4 antibody follows from the fact that the TSP Ab-4 antibody was used below to successfully detect thrombospondin fragments of interest in the plasma, including the plasma of cancer patients. Significantly, this TSP Ab-4 antibody is described as binding the collagen type V binding domain of thrombospondin.

30 For references regarding a fibrinogen-binding region within a 210-kDa fragment of TSP composed of three 70-kDa fragments that contain the region of interchain disulfide bonds, the procollagen homology region, and the type 1 and type 2 repeats, see p.24 of Adams et al. The Thrombospondin Gene Family; citation 53 therein, which is Lawler J et al.

Thrombin and chymotrypsin interactions with thrombospondin. *Ann N Y Acad Sci.* 1986;485:273-87; and citations immediately below. Additional references for the fibrinogen-binding regions to be excluded include: for a region in an 18-kDa amino-terminal heparin-binding domain of thrombospondin (so-called TSP18), see Bonnefoy A et al.: A 5 model of platelet aggregation involving multiple interactions of thrombospondin-1, fibrinogen, and GPIIbIIIa receptor. *J Biol Chem.* 2001 Feb 23;276(8):5605-12. For a region corresponding to synthetic peptide N12/I encompassing amino acid residues 151-164 of the N-terminal domain of thrombospondin-1, see Voland C et al.: Platelet-osteosarcoma cell interaction is mediated through a specific fibrinogen-binding sequence located within the N- 10 terminal domain of thrombospondin 1. *J Bone Miner Res.* 2000 Feb;15(2):361-368. Citations for two fibrinogen-binding domains include p. 24 of Adams *et al.* The Thrombospondin Gene Family (and citations 51-54 therein), and for the role of the type 1 15 repeats include Panetti TS et al.: Interaction of recombinant procollagen and properdin modules of thrombospondin-1 with heparin and fibrinogen/fibrin. *J Biol Chem.* 1999 Jan 1;274(1):430-7.

Thrombospondin is a glycosylated protein. Therefore, depending on which portion of thrombospondin is considered, the thrombospondin fragments of the invention may be glycosylated or non-glycosylated. Potential sites for N-linked carbohydrate chains include N-230 (in the N-terminal domain), N-342 (in the procollagen homology domain), N-503 (in the type 1 repeat domain), N-690 (in the region between the type 2 and type 3 repeat 20 domains), N-1033 (in the C-terminal domain), and N-1049 (in the C-terminal domain). It is also understood that specific C- and O-linked saccharide attachments occur, particularly in the type 1 repeat domain (see Hofsteenge J, Huwiler KG, Macek B, Hess D, Lawler J, Mosher DF, Peter-Katalinic J: C-mannosylation and O-fucosylation of the thrombospondin 25 type 1 module. *J Biol Chem.* 2001 Mar 2;276(9):6485-6498). It is also understood that β -hydroxylation of thrombospondin can occur (such as at N-592, which is in the type 2 repeat domain; see Figure 2.2a in Adams JC et al. The Thrombospondin Gene Family, 1995, p. 16), and that any of these modifications can be incorporated, or not, into thrombospondin fragments and/or peptides of the current invention.

30 Nonglycosylated entities of particular interest are synthetic peptides.

In particular embodiments, the thrombospondin fragments of the invention are derivatized so that they comprise and/or are linked to a detectable label and/or a carrier. In particular embodiments, the label is selected from the group consisting of a radioactive label,

a fluorescent label, a chemical label, a colorometric label, an enzymatic label, a non-fluorescent label, a non-radioactive label, a biotin moiety, and an avidin moiety. In particular embodiments, the carrier is selected from the group consisting of a bead, a microsphere, a coded microsphere, a solid matrix, a keyhole limpet hemocyanin, an albumin, linkage to a cross-linking agent, an epitope tag, and an epitope.

It is understood that a synthetic or purified thrombospondin fragment of the invention retains its identity as a fragment of the invention even if it has been derivatized by the addition of additional material, such as detectable label, or through conjugation to another molecule, or by synthesizing it as part of a chimeric protein, to name just three of many possible examples.

Binding agents

The detection of either thrombospondin fragments or thrombspondin usually requires the use of agents that will bind to them. Such agents may be multi-chain antibodies, single-chain antibodies, proteins that are not antibodies, non-protein molecules, or derivatives or combinations thereof. Polyclonal and monoclonal antibodies are normally immunoglobulins, i.e., multi-chain antibodies. In the case of immunoglobulin-G (IgG), each antibody molecule consists of a pair of heavy chains and a pair of light chains. The multichain antibodies are typically from mammalian or avian sources. Single-chain antibodies and non-antbodies are discussed below.

The term "antibodies" by itself, when not specified as being a single-chain antibodies, refers to 4-chain antibodies, those with two heavy and two light polypeptide chains. By way of example, this includes but is not limited to the IgG classes of antibodies, but also other classes, such as ones that occur in higher multimers, such as IgM. IgA and IgY are also contemplated.

The term "protein" is intended to include not only molecules normally referred to as proteins but also those that may be referred to as polypeptides.

Methods of detecting the thrombspondin fragments while distinguishing, or not distinguishing, from thrombospondin itself

In one such an aspect, the invention includes an assay to detect a thrombospondin fragment of the invention wherein the assay distinguishes the thrombospondin fragment from thrombospondin itself. The thrombospondin fragments of particular interest are ones

found in humans and are within a range selected from the group consisting of 80 to 100kDa, 40 to 55 kDa and 20 to 30 kDa, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction. Most preferably they are selected from the group consisting of an ~ 85 kDa to 90 kDa fragment, an ~ 50 kDa fragment, and an ~ 30 kDa fragment. The assay may detect just one such fragment, or a combination of 2 or more.

In cases where the concentration of higher molecular weight forms, including thrombospondin itself, is low in a sample (such as in the samples shown in Figures 3 and 4, Results of Western Blot analysis using TSP Ab-4 antibody), detection of fragments without necessarily excluding thrombospondin is an approach also contemplated by the current invention. Low concentrations of thrombospondin can be achieved in many cases by preventing or reducing platelet activation during sample collection and/or storage (see below for contemplated methods). This aspect of the current invention comprises several advantages over conventional detection methods that have used binding agents against the entire thrombospondin molecule (and these binding agents have been limited to antibodies). Said advantages include but are not limited to the use of binding agents that are directed specifically against the fragments of interest and not portions of the thrombospondin molecule outside of these fragments, the use of relevant peptides and/or thrombospondin fragments to generate said binding agents (such as antibodies), the use of relevant peptides and/or thrombospondin fragments as assay calibrators, and the use of relevant peptides and/or thrombospondin fragments as assay indicators.

Any of several acceptable approaches can be used for the assay of a thrombospondin fragment (or fragments) wherein the assay distinguishes it from thrombospondin, and more than one of these can be used in a given assay. In one approach, the assay comprises a step wherein the fragment is physically separated from the thrombospondin. Generally that approach is combined with a step in which the presence of the fragment or thrombospondin is shown by their reaction with a specific binding agent. In particular embodiments, the physical separation technique is selected from the group consisting of gel electrophoresis, dialysis, chromatography, size chromatography, affinity chromatography, immunoaffinity chromatography, adsorption, immunoadsorption, isoelectric focusing, mass spectrometry, centrifugation, sedimentation, floatation, precipitation, immunoprecipitation, and gel filtration.

In a second approach, the assay distinguishes the fragment (or fragments) based on one or more epitopes (here "epitope" meaning a target to which a binding agent, *i.e.*, an

antibody or a non-antibody, binds) in the fragment that are not present in thrombospondin, including but not limited to an epitope at an end of a fragment and an epitope that is displayed by a fragment but is shielded in thrombospondin.

In a third approach, the assay distinguishes the fragment (or fragments) based on one or more epitopes in thrombospondin that are not present in the fragment. As an illustrative but not restrictive example, an epitope shared by thrombospondin and a thrombospondin fragment is used to obtain a quantitation of a total, thrombospondin plus thrombospondin fragment(s), from which is then subtracted a quantitation of thrombospondin obtained using an epitope present in thrombospondin but not present in a fragment. The difference between the two quantitations is a quantitation of the amount of fragment. As an example, epitopes in thrombospondin but not in at least one fragment from the group of an 80 to 100 kDa, a 40 to 55 kDa, or a 20 to 35 kDa fragment present in plasma can be selected from the group consisting of an epitope from outside the protease-resistant central core domain, an epitope in the N-terminal domain, an epitope in the N-terminal heparin-binding domain, a heparin-binding sequence in the N-terminal domain, a heparin-binding sequence in the N-terminal domain selected from the group consisting of residues 23-32 (RKGSGRRLVK), residues 23-29 (RKGSGRR), and residues 77-83 (RQMKKKTR) of the mature protein (see Chapter 2, "The primary structure of the thrombospondins" in The Thrombospondin Gene Family by JC Adams, RP Tucker, & J Lawler, Springer-Verlag: New York, 1995, pp. 11-42, but especially p. 13 & Table 2.1; Chapter 6, "Mechanistic and functional aspects of the interactions of thrombospondins with cell surfaces," *ibidem* pp. 105-157, but especially pp. 108 & 114; Lawler J et al. Expression and mutagenesis of thrombospondin. *Biochemistry*. 1992 Feb 4;31(4):1173-80; and Cardin AD & Weintraub HJ. Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis*. 1989 Jan-Feb;9(1):21-32), a heparin-binding sequence in the N-terminal domain selected from the group consisting of residues 22-29 (ARKGSGRR), residues 79-84 (MKKTRG), and residues 178-189 (RLRIAKGGVNDN) of the mature protein (reviewed in the Discussion section of Voland C et al.: Platelet-osteosarcoma cell interaction is mediated through a specific fibrinogen-binding sequence located within the N-terminal domain of thrombospondin 1. *J Bone Miner Res.* 2000 Feb;15(2):361-368), an epitope in the C-terminal domain, an epitope in the C-terminal cell-binding domain, a thrombospondin epitope not found in a plasma fragment, a thrombospondin epitope not found in a plasma fragment of 80 to 100 kDa, a thrombospondin epitope not found in a plasma fragment of 40 to 55 kDa, and a thrombospondin epitope not

found in a plasma fragment of 20 to 35 kDa, where all kDa molecular weights are those after reduction. It is understood that the absence of a strong, functional heparin-binding domain from a thrombospondin fragment in plasma will be a factor allowing its accumulation in plasma (many heparin- or heparan-binding proteins are cleared from plasma very quickly; 5 see for example, Wallinder L et al. Rapid removal to the liver of intravenously injected lipoprotein lipase. *Biochim Biophys Acta*. 1979 Oct 26;575(1):166-73).

The epitopes may be divided into three Groups. Group 1: An epitope shared by thrombospondin and a thrombospondin fragment present in plasma is preferably one that is contained within an amino acid sequence selected from the group consisting of TEENKE (SEQ ID NO:1), CLQDSIRKVTEENKE (which includes an N-terminal Cys added to aid conjugation) (SEQ ID NO:2), LQDSIRKVTEENKE (SEQ ID NO:3), EGEARE (SEQ ID NO:4), PQMNGKPCEGEARE (SEQ ID NO:5), EDTDLD (SEQ ID NO:6), YAGNGIICGEDTDLD (SEQ ID NO:7), CNSPSPQMNGKPCEGEAR (SEQ ID NO:8), RKVTEENKELANELRRP (SEQ ID NO:9), CRKVTEENKELANELRRP (SEQ ID NO: 10), PQMNGKPCEGEAR (SEQ ID NO:11), CEGEAR (SEQ ID NO:12), RKVTEENKE (SEQ ID NO:13), or a portion at least 3 amino acyl residues in length (preferably at least 4 amino acyl residues in length, more preferably at least 6 amino acyl residues) of such an amino acid sequence.

Group 2: An epitope in thrombospondin but not in an 80 to 100 kDa, 40 to 55 kDa, 20 and/or 20 to 35 kDa fragment present in plasma is preferably one contained within an amino acid sequence selected from the group consisting of TERDDD (SEQ ID NO: 24), DFSGTFFINTERDDD (SEQ ID NO: 25), ERKDHS (SEQ ID NO: 26), TRGTLLALERKDHS (SEQ ID NO: 27), CTRGTLLALERKDHS (SEQ ID NO: 28) (which includes an N-terminal Cys added to aid conjugation), DDKFQD (SEQ ID NO: 29), 25 ANLIPPVPDDKFQD (SEQ ID NO: 30), CANLIPPVPDDKFQD (SEQ ID NO: 31) (which includes an N-terminal Cys added to aid conjugation), DCEKME (SEQ ID NO: 32), EDRAQLYIDCEKMEN (SEQ ID NO: 33) (although it is understood that this sequence and its fragments impinge on the sequence of the fibrinogen-binding N12/I peptide), CGTNRIPESGGDNSVFD (SEQ ID NO: 34), NRIPESGGDNSVFD (SEQ ID NO: 35), 30 GWKDFTAYRWRLSHRPKTG (SEQ ID NO: 36), CGWKDFTAYRWRLSHRPKTG (SEQ ID NO: 37) (which includes an N-terminal Cys added to aid conjugation), or a portion at least 3 amino acyl residues in length (preferably at least 4 amino acyl residues in length, more preferably at least 6 amino acyl residues) of such an amino acid sequence.

Various modifications, such as a C-terminal Cys, can be added to a peptide of interest to allow easier conjugation to a carrier protein such as KLH, ovalbumin, and others. This is particularly true for the following peptides: RKVTEENKELANELRRP (SEQ ID NO: 9), LQDSIRKVTEENKE (SEQ ID NO: 3); TRGTLALLALERKDHS (SEQ ID NO: 27), 5 and ANLIPPPVPDDKFQD (SEQ ID NO: 30), and these modifications provide alternative conjugation strategies for NRPESGGDNSVFD (SEQ ID NO: 35) and others.

In approaches related to the above, the assay can distinguish fragments from each other, based on physical separation methods and/or on shared and/or non-shared binding agent targets. Thus, for example, size-exclusion chromatography and/or SDS-10 polyacrylamide gel electrophoresis can be used to separate the ~85 to 90, ~50-, and ~30-kDa fragments from each other, for separate quantitation (an example of this is shown in Figure 3, with the quantitation presented in Table 2). Also, for example, an epitope (meaning a binding agent target) in the ~85 to 90-kDa fragment that is not contained in the ~50- and/or the ~30-kDa fragments can be used to assay it separately, and/or can be used to subtract its 15 contribution from a total to obtain results reflective of the smaller fragments.

Group 3: An additional epitope, useful as a binding agent target for distinguishing a fragment from full-length TSP, and/or distinguishing two fragments of different sizes is preferably one contained within an amino acid sequence selected from the group consisting of DDDDNDKIPDDRDNC (SEQ ID NO: 14), DDDDNDKIPDDRDNC[NH2] (SEQ ID 20 NO: 15), DDDDNDK (SEQ ID NO: 16), NLPNSGQEDYDKDG (SEQ ID NO: 17), CNLPNSGQEDYDKDG (SEQ ID NO: 18), EDYDKD (SEQ ID NO: 19), CPYNHNPQADTDNNGEVD (SEQ ID NO: 20), CRLVPNPDQKDSGD (SEQ ID NO: 21), DQKDSGD (SEQ ID NO: 22), CPYVPNANQADHDKGKGDA (SEQ ID NO: 23), or a portion at least 3 amino acyl residues in length (preferably at least 4 amino acyl residues 25 in length, more preferably at least 6 amino acyl residues) of such an amino acid sequence.

It is also understood that some peptides that contain an epitope shared by thrombospondin and a first thrombospondin fragment present in plasma may contain an epitope that is not shared by a second thrombospondin fragment present in plasma. Said peptides are useful in many applications described herein, including but not limited to 30 distinguishing thrombospondin from said second thrombospondin fragment, distinguishing said first from said second thrombospondin fragment, detecting and/or quantitating thrombospondin, detecting and/or quantitating said first thrombospondin fragment, detecting and/or quantitating said second thrombospondin fragment (in a combination assay described

elsewhere herein), and producing a binding agent. Said peptides, which form a subset of Group 1, can be selected from the group consisting of EGEARE (SEQ ID NO: 4), PQMNGKPCEGEARE (SEQ ID NO: 5), EDTDLD (SEQ ID NO: 6), YAGNGIICGEDTDLD (SEQ ID NO: 7), CNSPSPQMNGKPCEGEAR (SEQ ID NO: 8),
5 PQMNGKPCEGEAR (SEQ ID NO: 11), CEGEAR (SEQ ID NO: 12), or a portion at least 3 amino acyl residues in length (preferably at least 4 amino acyl residues in length, more preferably at least 6 amino acyl residues) of such an amino acid sequence.

It is also understood that the current invention also includes antibody and non-antibody molecules that bind these peptides, other peptides of thrombospondin specified herein, fragments thereof, and peptides that contain fragments thereof; as well as assays 10 using a reagent from this list. It is understood that an antibody or a non-antibody that distinguishes thrombospondin from a fragment, or one fragment from another, can be employed to affinity-purify thrombospondin or a fragment.

In embodiments of particular interest, a sample of material (liquid tissue, solid tissue, 15 urine, perspiration, cerebrospinal fluid, a body fluid, blood or a blood component, or stool; most preferably blood plasma) is taken or gathered from an organism (either a human or a non-human, preferably a mammal or a bird in the case of non-humans) and is subject to the assay. The inventions disclosed herein not only apply to fragments of human thrombospondin, but also to fragments of non-human thrombospondin. For example, there is 20 a need to detect the presence of or monitor the status of disease, such as a cancer, in livestock, racehorses, pets, and other economically and/or emotionally important animals. The current inventions meet these needs.

In one set of embodiments, the assay detects the presence of, or monitors the course 25 of, diseases and conditions that can affect plasma levels of thrombospondin fragments. Such diseases include, but are not limited to, many that in the prior art were assumed to affect plasma levels of thrombospondin: a cancer, renal failure, renal disease, atopic dermatitis, vasculitis, acute vasculitis, renal allograft, allergic asthma, diabetes mellitus, myocardial infarction, liver disease, splenectomy, dermatomyositis, polyarteritis nodosa, systemic lupus erythematosus, lupus erythematosus, Kawasaki syndrome, non-specific vasculitis, juvenile 30 rheumatoid arthritis, rheumatoid arthritis, vasculitis syndrome, Henoch-Schönlein purpura, thrombocytopenic purpura, purpura, an inflammatory condition, a condition associated with clotting, a condition associated with platelet activation, a condition associated with intravascular platelet activation, a condition associated with consumption of platelets,

heparin-induced thrombocytopenia, disseminated intravascular coagulation, intravascular coagulation, extravascular coagulation, a condition associated with endothelial activation, a condition associated with production and/or release of thrombospondin and/or a thrombospondin fragment, urticaria, hives, angioedema, a drug reaction, an antibiotic reaction, an aspartame reaction, atopic dermatitis, eczema, hypersensitivity, scleroderma, conditions associated with plugging of vessels, a condition associated with a cryofibrinogen, a condition associated with a cryoglobulin, and a condition associated with an anti-cardiolipin antibody.

In embodiments of particular interest, the assay for thrombospondin fragments is done to detect the presence of, or monitor the status of, a cancer in a human and/or in a non-human animal. In additional embodiments of interest, the assay is done to measure the degree of platelet activation.

In measurements of plasma levels of the fragments, it is preferred that the plasma is obtained by a method that prevents or reduces platelet activation and/or activation of a component of the clotting cascade during sample collection and/or storage; and/or by a method that prevents or reduces cleavage of thrombospondin into fragments (or fragments into smaller fragments) during sample collection and/or storage. Platelet activation and/or activation of a component of the clotting cascade during sample collection and/or storage can result in the release of thrombospondin, but also activation of proteases (including but not limited to a protease of the clotting cascade) that can cleave thrombospondin and some thrombospondin fragments, thereby complicating the assay. To prevent or reduce platelet activation during sample collection and/or storage, the method may be one that does not comprise the use of a tourniquet. Also to prevent or reduce platelet activation and/or activation of clotting during sample collection and/or storage, the method may, for example, comprise a step selected from the group consisting of: (1) use of a large-bore needle, (2) discarding of the initial portion of the collected blood, (3) use of a coated needle, (4) use of a coated tubing, (5) storage of sample between -1°C and 5°C, and (6) separation of plasma within 30 minutes of sample collection. Also to prevent or reduce platelet activation and/or protease activity during sample collection and/or storage, the method may comprise the use of an agent the use of an agent selected from the group consisting of a platelet inhibitor, a protease inhibitor, a serine protease inhibitor, an enzyme inhibitor, an inhibitor of an enzyme that is divalent cation dependent, a heparin, a heparin fragment, a heparan, an anticoagulant, a COX inhibitor, an inhibitor of a cell-adhesion molecule, an inhibitor of a surface receptor,

a glycoprotein inhibitor, an inhibitor of a glycoprotein IIb/IIIa receptor, a thrombin inhibitor, an inhibitor of degranulation, a chelator, a citrate compound, theophylline, adenosine, and dipyridamole (Diatube H vacutainers containing citrate, theophylline, adenosine, and dipyridamole are commercially available from Becton Dickinson; see Bergseth G et al. A 5 novel enzyme immunoassay for plasma thrombospondin: comparison with beta-thromboglobulin as platelet activation marker in vitro and in vivo. *Thromb. Res.* 99:41-50, 2000). Devices that minimize platelet activation and/or protease activity in a sample are also contemplated and include, but are not limited to, a collection tube containing a cocktail of platelet and/or clotting inhibitors, a collection tube containing a protease inhibitor, a 10 collection tube containing an inhibitor of a protease that is or is derived from a blood component, and a device that discards or allows the easy discarding of the initial portion of collected blood. These methods can also be applied to samples of other body fluids.

A related aspect of the invention is a combination diagnostic test (especially for cancer) comprising at least two types of diagnostic tests, one of said tests being the assay for 15 a thrombospondin fragment (or fragments) or a portion (or portions) thereof in plasma, the other assay not being based on a thrombospondin fragment or portion. In one set of embodiments, the test not based on a thrombospondin fragment or portion thereof is selected from the group consisting of an imaging test, a radiographic test, a nuclear medicine test, a magnetic resonance imaging test, a blood test, a biopsy, a genetic test, a guaiac test, a test for 20 fecal occult blood, and a test for fecal blood, a cancer test not based on a thrombospondin fragment or portion thereof, a disease test not based on a thrombospondin fragment or portion thereof, and an endoscopy. In particular embodiments of the foregoing methods, a thrombospondin fragment comprises a detectable label (at least during some part of the method).

25 Detection can, for example, be part of a screening process. Such a screening could include a comparison against a reference value, involve a comparison against a previous value from the same individual; and/or be done repeatedly and/or periodically (e.g., once a year, once every six months, or once every 2, 3, 4, 5 or 10 years.). It is understood that screening can be performed on humans and/or on non-human animals

30 The foregoing methods are assays to detect a thrombospondin fragment of the invention wherein the assay distinguishes, or does not distinguish, a thrombospondin fragment from thrombospondin, or one thrombospondin fragment from another thrombospondin fragment. In any case, such fragments can be referred to as "target"

fragments for purposes of the assay. In many instances it is desirable to have the method also comprise a calibration step or procedure, in which known amounts of a thrombospondin fragment (such as a peptide) are subjected to the method. Such "calibration" fragments are optionally detectably labeled. It is possible to perform the assays in which the target and 5 calibration fragments comprise different detectable labels (or where one is detectably labeled and the other is not).

It is understood that interference resulting from fibrinogen binding to an N-terminal domain of thrombospondin is unlikely to affect the detection of thrombospondin fragments related to the protease-resistant core domain (which lack the N-terminal domain). 10 Nevertheless, assays of thrombospondin could be affected (thus, avoiding that region of the N-terminus when assaying thrombospondin and/or diluting, removing, inhibiting, and/or otherwise compensating for interfering molecules is contemplated).

Additional potentially interfering substances, inferred from reports that these molecules are present in plasma and that they bind TSP, are plasminogen, histidine rich 15 proteins including histidine-rich glycoprotein, and fibronectin (See, for example, Walz DA et al., Semin Thromb Hemost. 13(3):317-025 (1987); Vanguri VK et al., Biochem J. 2000 Apr 15; 347(Pt 2):469-73). For binding of histidine-rich glycoprotein, two regions of thrombospondin have been implicated: type 1 repeats (Simantov et al. J Clin Invest. 2001 20 Jan, 107(1):45-52) and a TSP heparin binding domain (Vanguri VK et al., 2000). The heparin-binding domain of thrombospondin is expected to be absent from the circulating fragments.

To compensate for interfering substances in assays for thrombospondin fragments, diluting, removing, inhibiting, and/or otherwise compensating for interfering molecules is contemplated. As an illustrative, but not limiting, example, the inclusion of an inhibitor of 25 thrombospondin-fibrinogen interactions is contemplated. Such an inhibitor is selected from the group consisting of synthetic peptide N12/I encompassing amino acid residues 151-164 of the N-terminal domain of thrombospondin-1 (see Voland C et al.: Platelet-osteosarcoma cell interaction is mediated through a specific fibrinogen-binding sequence located within the N-terminal domain of thrombospondin 1. J Bone Miner Res. 2000 Feb;15(2):361-8), and 30 an antibody to the cyanogen bromide cleavage fragment composed of residues 241-476 of the carboxyl-terminal end of the alpha chain of fibrinogen (see Tuszyński GP et al.: The interaction of human platelet thrombospondin with fibrinogen. Thrombospondin purification and specificity of interaction. J Biol Chem. 1985 Oct 5;260(22):12240-5).

Single chain antibodies and non-antibodies

Raising conventional antibodies (also referred to herein simply as "antibodies" as opposed to "single chain antibodies"; and an example of a conventional antibody is IgG, 5 which is composed of two heavy chains and two light chains) is merely one of a number of methods that are generally based on the approach of random, semi-random, directed, combinatorial, and/or other means for the generation of large numbers of diverse peptides and/or non-peptides, that is then followed by a selection procedure to identify within this large number those peptides and/or non-peptides that bind to a target and/or an epitope 10 within a target. Selection can then be followed by methods for improving the peptides and/or non-peptides to achieve better affinity and/or specificity. These diverse peptides and/or non-peptides may be conventional multi-chain antibodies (polyclonal or monoclonal), single-chain antibodies, or non-antibodies, including but not limited to peptides, products of phage display, aptamers, DNA, RNA, or modified DNA or RNA. Also contemplated are 15 thrombospondin receptors and/or binding proteins (such as a CSVTG receptor, a CSVTG binding molecule, CD36, angiocidin, 26S proteasome non-ATPase regulatory subunit 4, and/or anti-secretory factor).

A well-known procedure for generation of large numbers of diverse peptides is through phage display, which is then followed by selection and can be further refined 20 through other techniques such as molecular evolution (see, for example, Flores-Flores, C. et al, Development of human antibody fragments directed towards synaptic acetylcholinesterase using a semi-synthetic phage display library. *J Neural Transm Suppl.* 2002;(62):165-179; Qian, M.D, et al, Anti GPVI human antibodies neutralizing collagen-induced platelet aggregation isolated from a recombinant phage. *Human. Antibodies.* 25 2002;11(3):97-105). scFv constructs can be made by linking variable domains of heavy (VH) and light (VL) chains together via a polypeptide linker (for example, see Asvadi P et al. Expression and functional analysis of recombinant scFv and diabody fragments with specificity for human RhD. *J Mol Recognit* 15:321-330, 2002). Peptides generated then selected (and then possibly improved) via this approach have been used in ELISAs and 30 ELISA-like assays of their targets (e.g., see Schlattner U et al. Isoenzyme-directed selection and characterization of anti-creatine kinase single chain Fv antibodies from a human phage display library. *Biochim Biophys Acta.* 2002 Dec 12;1579(2-3):124-32; Oelschlaeger P et al. Fluorophor-linked immunosorbent assay: a time- and cost-saving method for the

characterization of antibody fragments using a fusion protein of a single-chain antibody fragment and enhanced green fluorescent protein. *Anal Biochem.* 2002 Oct 1;309(1):27; Nathan S et al. Phage display of recombinant antibodies toward *Burkholderia pseudomallei* exotoxin. *J Biochem Mol Biol Biophys.* 2002 Feb;6(1):45-53; Lu D et al. Fab-scFv fusion protein: an efficient approach to production of bispecific antibody fragments. *J Immunol Methods.* 2002 Sep 15;267(2):213-26; Zhang W et al. Production and characterization of human monoclonal anti-idiotype antibodies to anti-dsDNA antibodies. *Lupus.* 2002;11(6):362-9; Reiche N et al. Generation and characterization of human monoclonal scFv antibodies against *Helicobacter pylori* antigens. *Infect Immun.* 2002 Aug;70(8):4158-64; Rau D et al. Single-chain Fv antibody-alkaline phosphatase fusion proteins produced by one-step cloning as rapid detection tools for ELISA. *J Immunoassay Immunochem.* 2002;23(2):129-43; and Zhou B et al. Human antibodies against spores of the genus *Bacillus*: a model study for detection of and protection against anthrax and the bioterrorist threat. *Proc Natl Acad Sci U S A.* 2002 Apr 16;99(8):5241-6; Baek H et al., An improved helper phage system for efficient isolation of specific antibody molecules in phage display. *Nucleic Acids Res.* 2002 Mar 1; 30(5):e18).

scFv constructs can be based on antibodies, as in most of the references above, on T-cell receptors (e.g., Epel M et al. A functional recombinant single-chain T cell receptor fragment capable of selectively targeting antigen-presenting cells. *Cancer Immunol Immunother.* 2002 Dec;51(10):565-573), or on other sequences. Different phage coat proteins have been used to display the diverse peptides (see Gao C et al. A method for the generation of combinatorial antibody libraries using pIX phage display. *Proc Natl Acad Sci USA.* 2002 Oct 1;99(20):12612-6). For an example of fusion constructs, see Lu D et al. Fab-scFv fusion protein: an efficient approach to production of bispecific antibody fragments. *J Immunol Methods.* 2002 Sep 15;267(2):213-26.

For an example of molecular evolution to improve binding affinity, see Rau D et al. Cloning, functional expression and kinetic characterization of pesticide-selective Fab fragment variants derived by molecular evolution of variable antibody genes. *Anal Bioanal Chem.* 2002 Jan;372(2):261-7. Examples of other modifications "to improve affinity or avidity, respectively [include] by mutating crucial residues of complementarity determining regions or by increasing the number of binding sites making dimeric, trimeric or multimeric molecules." (the quote is from a review article, Pini A & Bracci L, Phage display of antibody fragments. *Curr Protein Pept Sci.* 2000 Sep;1(2):155-169). The initial set of diverse

molecules can be enriched by using sequences from animals or humans exposed to or expressing antibodies against the target (see again Zhang W et al. Lupus 2002; and Reiche N et al. Infect Immun 2002).

Single chain antibodies can also be generated by using *Escherichia coli* (see Sinacola JR & Robinson AS, Rapid folding and polishing of single-chain antibodies from *Escherichia coli* inclusion bodies, *Protein Expr Purif.* 2002 Nov; 26(2):301-308.)

Non-antibodies also include aptamers and non-antibodies that comprise aptamers. Aptamers are DNA or RNA molecules that have been selected (e.g., from random pools) on the basis of their ability to bind to another molecule (discussed for example at the web site of the Ellington lab, in the Institute of Cellular and Molecular Biology, at the University of Texas at Austin, <http://aptamer.icmb.utexas.edu/>), wherein said molecule can be a nucleic acid, a small organic compound, or a protein, peptide, or modified peptide (such as thrombospondin or a portion thereof.). An aptamer beacon is an example of a non-antibody that comprises an aptamer (See Hamaguchi N et al., Aptamer beacons for the direct detection of proteins. *Anal. Biochem.* 2001 Jul 15;294(2):126-131.)

Angiocidin is a CSVTG-specific tumor cell adhesion receptor, see patent application WO 0105968, also NCBI protein accession number CAC32386.1 and/or CAC32387.1 (corresponding to nucleotide accession numbers AX077201 and AX077202), the amino acid sequences specified by those two protein accession numbers as of the date of filing of this application being incorporated herein by reference. It is understood that anti-secretory factor cDNA contains essentially identical nucleotide sequence (e.g., accession # U24704, 99% match by BLAST alignment) to that of angiocidin, as does the nucleotide sequence for the proteasome (prosome, macropain) 26S subunit, non-ATPase, 4 (PSMD4; e.g., accession # NM_002810, also 99% match by BLAST). Anti-secretory factor has the same amino acid sequence as angiocidin, except that AX077201 has a 9-bp insert compared to AX077202, which would mean an additional three amino acyl residues in the encoded protein. Thus, the terms herein are used interchangeably. The NCBI summary for NM_002810 is as follows: "The 26S proteasome is a multicatalytic proteinase complex with a highly ordered structure composed of 2 complexes, a 20S core and a 19S regulator. The 20S core is composed of 4 rings of 28 non-identical subunits; 2 rings are composed of 7 alpha subunits and 2 rings are composed of 7 beta subunits. The 19S regulator is composed of a base, which contains 6 ATPase subunits and 2 non-ATPase subunits, and a lid, which contains up to 10 non-ATPase subunits. Proteasomes are distributed throughout eukaryotic

cells at a high concentration and cleave peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway. An essential function of a modified proteasome, the immunoproteasome, is the processing of class I MHC peptides. This gene encodes one of the non-ATPase subunits of the 19S regulator lid. Two alternate transcripts encoding two 5 different isoforms have been described. Pseudogenes have been identified on chromosomes 10 and 21. Transcript Variant: This variant (1) encodes the longer protein (isoform 1)." Other names for the protein from the protein accession file ([NP_002801.1](#)) include "proteasome 26S non-ATPase subunit 4 isoform 1; antisecretory factor 1; 26S protease subunit S5a; S5a/antisecretory factor protein; multiubiquitin chain binding protein; 26S 10 proteasome non-ATPase regulatory subunit 4".

Methods of producing antibodies against the fragments of the invention

In another general aspect, the invention is a method of producing antibodies against 15 an above-noted thrombospondin fragment and/or portion thereof, the method comprising administering such a fragment or portion to an organism (especially a mammal or a bird) capable of producing antibodies. It is understood that said antibodies may comprise monoclonal antibodies and/or polyclonal antibodies. For monoclonal antibodies it is understood that cells from the organism are typically used in the production of hybridomas. 20 For production of antibodies, including monoclonal antibodies, it is understood that any of the thrombospondin fragments and/or portions can be conjugated to a carrier molecule, including but not limited to keyhole limpet hemocyanin and bovine serum albumin, to facilitate the antibody response.

A cell and a cell line for producing the aforementioned monoclonal antibodies are 25 aspects of the invention. Examples of such cells include, but are not limited to, hybridomas, transfected cell lines, and infected cells.

Kits of the invention

Kits related to the above inventions are themselves aspects of the invention. Such kits 30 are, for example, those that facilitate the determination of the presence of, and/or the amount of, and/or the concentration of, a thrombospondin fragment or fragments in a material taken or gathered from an organism. Such kits optionally comprise a thrombospondin fragment or fragments, or a portion or portions thereof, of the invention. Such kits can comprise a

binding agent or agents specific for a thrombospondin fragment, or portion thereof, of interest. They optionally comprise binding agents that will react with thrombospondin but not a fragment or fragments, and/or a portion or portions thereof, of interest. They optionally comprise binding agents that distinguish between thrombospondin and a fragment, and/or between one fragment and another. If intended for solid tissue, the kits 5 may comprise a homogenizing means for extracting a fragment into a solution, which optionally may also be provided. Binding agents of the current invention can also be used for other well-known detection methods, including but not limited to immunohistochemistry.

Preferred binding agents are proteins, although non-proteins are also contemplated. 10 Such proteins include both antibodies and nonantibodies.

Optionally, the kits comprise a means for separating or distinguishing a fragment or fragments (or portions thereof) from thrombospondin. The kits can also include a thrombospondin fragment, a peptide derived from such fragment, or a derivatized fragment or peptide, to facilitate detection and calibration.

15 In one set of embodiments, the kits are adapted for use in an automated assay, such as one using a clinical autoanalyzer.

Particular kit aspects of the invention can also be summarized as follows:

20 A kit for the determination of the presence of, and/or the amount of, and/or the concentration of, a thrombospondin fragment or fragments in a material taken or gathered from an organism, said kit comprising a thrombospondin fragment or portion thereof.

A kit for the determination of the presence of, and/or the amount of, and/or the concentration of, one or more thrombospondin fragments in a material taken or gathered from an organism, said kit comprising a binding agent capable of binding said one or more fragments.

25 Particular embodiments are:

Such kits wherein the binding agent comprises a protein.

Such kits wherein said protein comprises an antibody.

Such kits wherein the antibody is a monoclonal antibody or a polyclonal antibody.

Such kits wherein said protein comprises a fragment of an antibody.

30 Such kits wherein said protein comprises a single-chain antibody.

Such kits wherein said single chain antibody is derived from a phage display library.

Such kits wherein said protein is a non-antibody, the non-antibody being a protein that is neither a multi-chain antibody nor a single-chain antibody.

Such kits wherein said protein non-antibody is selected from the group consisting of a thrombospondin receptor, a thrombospondin receptor that binds within a protease-resistant core region, a thrombospondin receptor that binds a TSP fragment present in the plasma of a cancer patient, a CSVTCG receptor, a CSVTCG binding molecule, a CD36 (which 5 reportedly binds CSVTCG; see Carron JA et al., A CD36-binding peptide from thrombospondin-1 can stimulate resorption by osteoclasts in vitro. *Biochem Biophys Res Commun.* 2000 Apr 21;270(3):1124-7), angiocidin, anti-secretory factor, 26S proteasome non-ATPase regulatory subunit 4, fragments thereof that bind to their respective targets, and combinations, chimeras, and recombinant versions of said receptors and fragments.

10 Such kits wherein said binding agent comprises a non-protein.
Such kits wherein said binding agent comprises an aptamer.
Such kits wherein said binding agent comprises angiocidin, anti-secretory factor, and/or 26S proteasome non-ATPase regulatory subunit 4.
Other particular kit aspects of the invention can be summarized as follows:

15 A kit for the determination of the presence of, and/or the amount of, and/or the concentration of, one or more thrombospondin fragments in a material taken or gathered from an organism, said kit comprising a binding agent that will react with thrombospondin but not with a fragment of interest. Particular embodiments are:
Such kits wherein said binding agent comprises a protein;
20 Such kits wherein said protein comprises an antibody;
Such kits wherein said antibody is a monoclonal antibody or a polyclonal antibody;
Such kits wherein said protein comprises a fragment of an antibody;
Such kits wherein said protein comprises a single-chain antibody;
Such kits wherein said single chain antibody is derived from a phage display library;
25 Such kits wherein the protein is a non-antibody, the non-antibody being a protein that is neither an antibody nor a single-chain antibody;
Such kits wherein said non-antibody is selected from the group consisting a an integrin, an RGD receptor, an RFYVVMWK receptor, an RFYVVM receptor, an FYVVMWK receptor, an IRVVM receptor, fragments thereof that bind to their respective 30 targets, and combinations, chimeras, and recombinant versions of said receptors, integrins, and fragments; and
Such kits wherein said binding agent comprises an aptamer, meaning a DNA or RNA or related compound, that binds thrombospondin or a thrombospondin fragment.

Such kits wherein said binding agent comprises angiocidin, anti-secretory factor, and/or 26S proteasome non-ATPase regulatory subunit 4.

Several motifs within thrombospondin for binding to many of the receptors referred to above are shown in in Figure 2.2a of Adams, J.C., et al., *The thrombospondin Gene Family*, Springer Verlag, New York, 1995, p. 16. A CSVTG receptor, a CSVTG binding molecule, an angiocidin, an anti-secretory factor, a CD36, and/or fragments and derivatives thereof will be useful for assaying a thrombospondin fragment in a cancer patient.

Focus on Neoplastic Disease

10 The invention as it pertains to the detection or monitoring of neoplastic disease can also be summarized as the following:

A method to detect the presence of neoplastic disease in an individual, wherein the method comprises the steps of:

- (1) measuring the individual's plasma level of a thrombospondin fragment;
- 15 (2) utilizing the result of step (1) in a diagnosis as to whether the individual has a neoplastic disease; said fragment being at least 6 contiguous amino acyl residues in length but less than 110 kDa (preferably less than 100 kDa).

Related is such a method, where the individual referred to therein is a first individual and wherein the method further comprises the steps of:

20 (3) measuring a second individual's plasma level of the thrombospondin fragment, said second individual considered to not have neoplastic disease;

(4) utilizing the result of step (3) is the diagnosis of whether the first individual has a neoplastic disease. For example, such a method wherein the greater the extent to which the first individual's plasma thrombospondin fragment level exceeds the plasma thrombospondin 25 level of the second individual, the more likely that the diagnosis will be that the first individual has a neoplastic disease and/or a neoplastic disease more advanced than that of the second person. It is also understood that values from the first individual taken over time can be compared with one another, to assess the likelihood of the appearance of disease and/or progression and/or regression of disease. Particular embodiments are:

30 Such methods wherein the fragment is selected from the group consisting of an ~85 to 90 kDa fragment, and ~50 kDa fragment, and an ~ 30kDa fragment, wherein the size in kDa is that determined by gel electrophoresis after disulfide bond reduction;

Such methods wherein the neoplastic disease is selected from the group consisting of an adenoma, adenocarcinoma, carcinoma, lymphoma, leukemia, and sarcoma;

Such methods wherein the neoplastic disease is an internal cancer;

Such methods wherein the neoplastic disease is selected from the group consisting of

5 a cancer of the respiratory system, a cancer of the circulatory system, a cancer of the musculoskeletal system, a cancer of a muscle, a cancer of a bone, a cancer of a joint, a cancer of a tendon or ligament, a cancer of the digestive system, a cancer of the liver or biliary system, a cancer of the pancreas, a cancer of the head, a cancer of the neck, a cancer of the endocrine system, a cancer of the reproductive system, a cancer of the male reproductive system, a cancer of the female reproductive system, a cancer of the genitourinary system, a cancer of a kidney, a cancer of the urinary tract, a skin cancer, a cancer of other sensory organs (such as eye, ear, nose, tongue), a cancer of the nervous system, a cancer of a lymphoid organ, a blood cancer, a cancer of a gland, a cancer of a mammary gland, a cancer of a prostate gland, a cancer of endometrial tissue, a cancer of mesodermal tissue, a cancer of ectodermal tissue, and a teratoma;

10

15

Such methods wherein the neoplastic disease is selected from the group consisting of a cancer of solid tissue, a cancer of the blood or the lymphatic system, a non-metastatic cancer, a premetastatic cancer, a metastatic cancer, a poorly differentiated cancer, a well-differentiated cancer, and a moderately differentiated cancer.

20 Such methods wherein the measurement of a plasma thrombospondin fragment level comprises the use of a binding agent, said binding agent being capable of binding said thrombospondin fragment (Such binding agents are discussed above in the context of the kits of the invention); and

25 In particular embodiments, the thrombospondin fragment is separated from thrombospondin before said fragment is bound to the binding agent.

Such methods wherein said method comprises the use of a binding agent, comprising a binding agent capable of binding thrombospondin but not the thrombospondin fragment. Possible binding agents are discussed above in the context of kits of the invention.

30 In particular embodiments, the thrombospondin fragment is separated from thrombospondin before said fragment is bound to the binding agent.

Related inventions are:

A method of producing antibodies against a thrombospondin fragment, said method comprising administering said fragment to an organism capable of producing antibodies;

Said method of producing antibodies wherein said fragment is at least 6 amino acyl residues in length but less than 110 kDa (preferably less than 95 kDa). A polyclonal 5 antibody preparation produced by said method;

A monoclonal antibody produced by said method;

A cell line producing said monoclonal antibody; and

A method of producing a binding agent against a thrombospondin fragment, said method comprising the use of phage display.

10 Said method of producing a binding agent, wherein said method comprises the selection of a thrombospondin-binding or thrombospondin fragment-binding phage from a phage display.

Said method of producing a binding agent, wherein said fragment at least 6 amino acyl residues in length.

15

Cancer detection method comprising measuring platelet activation

An additional general aspect of the invention is an assay for the presence of cancer in an organism, said method comprising measuring the extent of platelet activation.

20

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic drawing of thrombospondin.

Figure 2. Results of staining a gel with Coomasie Blue. Lanes, left to right are in the sequence: a lane with the molecular weight standards (Stds), followed by samples A to G.

25

Figure 3. Results of Western Blot analysis using TSP Ab-4 antibody and fluorescence detection. Lanes, left to right are in the sequence: a lane with the molecular weight standards (Stds), followed by samples A to G, which correspond to aliquots of the same samples as in Figure 2.

30

Figure 4. Analysis of the same samples as for Figure 3, using urea denaturation before electrophoresis, followed by electrophoresis through a 12% acrylamide gel and enzymatic colorometric detection after blotting.

DETAILED DESCRIPTION OF THE INVENTION

The terms "thrombospondin" and "thrombospondin-1" are used interchangeably herein. It is understood that a single "band" on an electrophoresis gel may in fact reflect the presence of a collection of fragments that together form a population that, during gel 5 electrophoresis under reducing conditions, electrophoreses at similar rates.

The terms "test" and "assay" are also used interchangeably.

A "purified" fragment is for example (1) one that is found in human plasma and that has been purified (for example has been isolated from gels on which the plasma has been electrophoresed). A purified fragment is not one that is in human plasma, or other part of a 10 human, and that has not undergone at least some degree of purification.

A "synthesized fragment" is, for example, one that has been synthesized in a laboratory (e.g., by recombinant DNA technology or by chemical synthesis) so as to have the primary structure of such a fragment or a portion thereof.

The amino acid sequence of human thrombospondin-1 from GenBank is:

15 ACCESSION NM_003246 (protein_id=NP_003237.1)
VERSION NM_003246.1 GI:4507484

MGLAWGLVLF LMHVC GTN RIPE SG GD NSVFD I FEL TGAARK GSG RRLVKG PDPSS
PAFRIEDANLIP VPDDK FQDLV DAVRAEKG FLLASLRQM KKTRG TLLA LERKDHS
20 GQVFSVV SNGKAGT LLDLSL TVQG KQH VVS VEE ALLATG QWKSITLFVQEDRA QLYI
DCEKMEN AELDVPIQSVFTRDL ASIARL RIAKGGVNDNFQGV LQNVRFVFGTT PEDI
LRNKGCS SSTS VLLTLDNNVNGSSPAIRTNYIGHKTKDLQAI CGISCDELSSMVLEL
RGLRTIVT TLQDSIRK VTEENK E LANE LRRPLCYHNGVQYRNNEEWTVDSCTECH
CQNSVTICKVSCPIM PCSNATVPDGECCPRCWP SDSADDGWSPWSEWTSCSTSCG
25 NGIQQRGRSCDS LNRCEGSSVQTRTCHIQECDKRFKQDGGW SHWSPWSSCSVT CG
DG VITRIRL C N S P S P Q M N G K P C E G E A R E T K A C K K D A C P I N G G W G P W S P W D I C S V T C
GGGVQKRSR L C N N P A P Q F G G K D C V G D V T E N Q I C N K Q D C P I D G C L S N P C F A G V K C T S
Y PDGSWKGACPPGYSGNGIQCTD V D E C K E V P D A C F N H N G E H R C E N T D P G Y N C L P
C P P R F T G S Q P F G Q Q G V E H A T A N K Q V C K P R N P C T D G T H D C N K N A K C N Y L G H Y S D P M Y
30 R C E C K P G Y A G N G I I C G E D T D L D G W P N E N L V C V A N A T Y H C K K D N C P N L P N S G Q E D Y
D K D G I G D A C D D D D D N D K I P D D R D N C P F H Y N P A Q Y D Y D R D D V G D R C D N C P Y N H N P
D Q A D T D N N G E G D A C A A D I D G D G I L N E R D N C Q Y V V N V D Q R D T D M D G V G D Q C D N C
35 P L E H N P D Q L D S D R I G D T C D N N Q D I D E D G H Q N N L D N C P Y V P N A N Q A D H D K D G K G
D A C D H D D D N D G I P D D K D N C R L V P N P D Q K D S D G D G R G D A C K D D F D H D S V P D I D D I C
P E N V D I S E T D F R R F Q M I P L D P K G T S Q N D P N W V V R H Q G K E L V Q T V N C D P G L A V G Y D E
F N A V D F S G T F F I N T E R D D D Y A G F V F G Y Q S S S R F Y V V M W K Q V T Q S Y W D T N P T R A Q G
Y S G L S V K V V N S T T G P G E H L R N A L W H T G N T P G Q V R T L W H D P R H I G W K D F T A Y R W R
L S H R P K T G F I R V V M Y E G K K I M A D S G P I Y D K T Y A G G R I G L F V F S Q E M V F F S D L K Y E C
R D P (SEQ ID NO: 38)

40

The underlined N in the first line of the sequence above refers to amino acid number 1 of the mature protein (*i.e.*, without the 18- to 22-residue signal peptide sequence, here assumed to be 18 residues; see p. 13 and Figure 1 in Adams JC *et al.* The Thrombospondin Gene Family, 1995).

5 Here is a partially annotated version of the human TSP-1 sequence from GenBank, broken into domains, and including indications of some of the functional regions that have been identified in the literature.

10 MGLAWGLGVLF LMHVCGT (SEQ ID NO: 39) [The signal peptide is considered to be 18-22 residues long (18 residues assumed here, following The Thrombospondin Gene Family by JC Adams *et al.* 1995)]

15 NRIPESGGDNSVFDI FELTGAARKGSGRRLVKGPD PSSPAFRIEDANLIPPV PDDKFQDLVD AVRAEKGFLL LASLRQMKKTRGTLLALERKDHSQVFSVVSNGKAGTLDSLTVQGKQHVVS VEEALLATGQWKSITLFVQEDRAQLYIDCEKMENAELDVPIQSVFTRDLASIARLRIA KGGV

20 15 NDNFQGVLQNVRFVFGTTPEDILRNKGCSSTS VLLTLDNNVVNGSSPAIRNY (SEQ ID NO: 40) [N-terminal domain (1-240). The underlined N at the beginning of this domain refers to amino acid number 1 of the mature protein (*i.e.*, without the 18- to 22-residue signal peptide sequence, here assumed to be 18 residues; see p. 13 and Figure 1 in Adams JC *et al.* The Thrombospondin Gene Family, 1995). Two apparent heparin-binding regions are double-underlined. Finally, the last underlined region in this domain corresponds to “synthetic peptide N12/I encompassing amino acid residues 151-164 of the N-terminal domain of TSP-1”, which was reported to bind fibrinogen.]

25 IGHKT KDLQAICGISCDELSSM (SEQ ID NO: 41) [Domain of inter-chain disulfide bonds (241-262)]

30 VLELRGLRTIVTTLQDSIRKVTEENKELANELRRPPLCYHNGVQYRNNEEWTVDSCTECHC QNSVTICKKVSCPIMPCSNATVPDGECCPRCWPSDSA (SEQ ID NO: 42) [Procollagen homology domain (263-360). Notice that the collagen V-binding region (valine[333] to lysine[412]), which is double underlined here, is partly in this domain and partly in the first type 1 repeat, which immediately follows this domain.]

DDGWSPWSEWTSCSTSCGNGIOQGRSCDSLNNRCEGSSVQRTCHIOECDKRFKQ
DGGWSHWSPWSSCSVTCGDGVITRIRLCNSPS PQMNGKPCEGEARETKACKKDACP I

NGGWGPWSPWDICSVTCGGGVQKRSRLCNNPAPQFGGKDCVGDVTENQICNKQDCPI (SEQ ID NO: 43) [Domain of type 1 repeats (361-530). This domain consists of three type 1 repeats. The double-underlined segment at the beginning of this domain is the continuation of the collagen V-binding region (valine[333] to lysine[412]).]

5 DGCLSNPCFAGVKCTSYPDGSWKCGACPPGYSGNGIQCTDV
DECKEVPDACPENHNGEHRCENTDPGYNCLPCPPRFTGSQPFQGQVEHATANKQVCKPR
NPCTDGTHDCNKNACKNYLGHYSDPMYRCECKPGYAGNGIICGE (SEQ ID NO: 44)
[Domain of type 2 repeats (531-673). This domain consists of three type 2 repeats.]
DTDLDGWPNENLVCVANATYHCKK (SEQ ID NO: 45) [Region between the type 2 and the
10 type 3 repeat (674-697)]
DNCPNLPNSGQEDYDKDGIGDACDDDDNDKIPDDR (SEQ ID NO: 46)
DNCPFHYNPAQYDYDRDDVGDRC (SEQ ID NO: 47)
DNCPYNHNPDQADTDNNGEGDACAADIDGDGILNER (SEQ ID NO: 48)
DNCQYVYNVDQRDTMDGVGDQC (SEQ ID NO: 49)
15 DNCPLEHNPDQLSDSDRIGDTCDNNQDIDEDGHQNNL (SEQ ID NO: 50)
DNCPYVPNANQADHDKGKGACDHDDNDGIPDDK (SEQ ID NO: 51)
DNCRLVPNPDQKDSGDGRGDACKDDFDHDSVPDID (SEQ ID NO: 52) [Domain of type
3 repeats (698-925). This domain consists of seven type 3 repeats.]
DICPENVDISETDFRRFQMIPLDPKGTSQNDPNVVVRHQGKELVQTVNCDPGLAVGYDEFN
20 AVDFSGTFFINTERDDDYAGFVFGYQSSSRFYVVMWKQVTQSYWDTNPTRAQGYSGLSVKV
VNSTTGPGEHLRNALWHTGNTPGQVRTLWHDPRHIGWKDFTAYRWRLSHRPKTGFIRVVMY
EGKKIMADSGPIYDKTYAGGRLGLFVFSQEMVFFSDLKYECRDP (SEQ ID NO: 53)
[C-terminal domain (926-1152)]

25 It is understood that genetic variants of thrombospondin exist, including but not limited to human polymorphisms (e.g., see dbSNP:[2229364](#), dbSNP:[2228261](#), dbSNP:[2292305](#), dbSNP:[2228262](#), and dbSNP:[2228263](#) for variants in the coding region; and dbSNP:[1051442](#), dbSNP:[3743125](#), dbSNP:[3743124](#), dbSNP:[1051514](#), dbSNP:[1131745](#), and dbSNP:[11282](#) for 3' UTR variants). The current invention contemplates assays that

detect polymorphic variants as well as common types involving the coding region, either through the use of an antibody or antibodies or other binding molecule or molecules that recognize variant and common peptide sequences, and/or through the use of sequences that are not polymorphic. It is understood that A-505 [alanine(505)] in the GenBank sequence 5 NM_003246 is instead given as a T [threonine(505)] in Figure 2.2a of Chapter 2, "The primary structure of the thrombospondins" in The Thrombospondin Gene Family by JC Adams, RP Tucker, & J Lawler, Springer-Verlag: New York, 1995, p. 16.

It is believed that the collagen type V binding domain corresponds to the region extending from valine(333) and lysine(412) of thrombospondin-1 (Takagi T *et al.* *J Biol Chem* 268:15544-15549, 1993; here, the residue numbers refer to the mature protein). Thus, 10 the collagen type V-binding region would include a portion of the procollagen homology region of thrombospondin and all or nearly all of the first type 1 repeat of thrombospondin (see Chapter 2, "The primary structure of the thrombospondins" in The Thrombospondin Gene Family by JC Adams, RP Tucker, & J Lawler, Springer-Verlag: New York, 1995, pp. 15 11-42, but especially p. 24). See Figure 1 of this application, as well as the annotated TSP sequence, above. As indicated on the Figure 1 of this application, the leftmost rectangle represents the N-terminal domain (mature residues 1 to ~240), which contains heparin-binding sequence; the short vertical lines represent Cys(252) and Cys(256) of human thrombospondin-1, which are involved in inter-chain disulfide bonds, to form trimers; the 20 first oval represents the procollagen homology domain (residues 263-360); the three slanted ovals represent the three type 1 repeats (residues 361-530), which resemble properdin and a malarial protein; the three tall ovals represent the three type 2 repeats (residues 531-673), which show similarities to the epidermal growth factor (EGF) repeat; there is a short sequence (residues 674-697) separating type 2 and type 3 repeats; the seven ovals represent 25 the seven type 3 repeats (residues 698-925), which are rich in aspartic acid and resemble the calcium-binding pocket of parvalbumin or calmodulin; and right-hand square represents the C-terminal cell-binding domain (residues 926 to the end, that is, Proline-1152; see Figure 2.2a in Adams JC *et al.* The Thrombospondin Gene Family, 1995, p. 16). The two chymotryptic fragments (70- and 50-kDa), and to some extent the 120-kDa tryptic fragment, 30 indicated schematically on Figure 1, correspond to the protease-resistant central core domain of thrombospondin.

Examples of cancers that can be detected using assays for the thrombospondin fragments include but are not limited to: adenoma, adenocarcinoma, carcinoma, lymphoma,

leukemia, sacrcoma, solid cancer, liquid cancer, metastatic cancer, pre-metastatic cancer, non-metastatic cancer, a cancer with vascular invasion, internal cancer, skin cancer, cancer of the respiratory system, cancer of the circulatory system, cancer of the musculoskeletal system, cancer of a muscle, cancer of a bone, cancer of a joint, cancer of a tendon or 5 ligament, cancer of the digestive system, cancer of the liver or biliary system, cancer of the pancreas, cancer of the head, cancer of the neck, cancer of the endocrine system, cancer of the reproductive system, cancer of the male reproductive system, cancer of the female reproductive system, cancer of the genitourinary system, cancer of a kidney, cancer of the urinary tract, cancer of a sensory system, cancer of the nervous system, cancer of a lymphoid 10 organ, a blood cancer, cancer of a gland (for example but not limited to cancer of a mammary or a prostate gland), cancer of an endometrial tissue, cancer of a mesodermal tissue, cancer of an ectodermal tissue, cancer of an endodermal tissue, a teratoma, a poorly-differentiated cancer, a well-differentiated cancer, and a moderately differentiated cancer.

One of the options for tests for the presence of thrombospondin fragments is to 15 fractionate the material (e.g., plasma) into fractions (e.g., positions on an electrophoresis gel, or chromatographic elution samples) collected by a technique capable of separating the fragments from thrombospondin (e.g., by electrophoresis, size-dependent chromatography, and/or affinity chromatography) and to detect the fragments in the fractions where such fragments would be expected to appear. Another of the various additional known options for 20 assays is to test the ability of plasma to inhibit the binding of thrombospondin fragments or portions thereof to compounds (e.g., antibodies) that specifically bind to them.

The thrombospondin fragments of primary interest in the diagnostic tests are ones that have apparent molecular weights of ~ 85 kDa (or ~ 90 kDa), ~ 50 kDa, and ~ 30 kDa as determined by SDS-PAGE electrophoresis after reduction (see Figures 3 and 4). Preferred 25 conditions for determining the molecular weights are those referred to below as "Standard Gel Electrophoresis Protocol." The assignment of a number such as 50 kDa to the size of a fragment reflects its approximate molecular weight as determined using the Standard Gel Electrophoresis Protocol.

It is believed that the ~85 kDa, ~50 kDa, and ~30 kDa fragments all contain an 30 immunogenic portion of "collagen type V-binding domain" of thrombospondin. In a preferred aspect of the invention, the fragments are detected by antibody that binds to such a domain, as is believed to be the case for the TSP Ab-4 monoclonal antibody referred to below. Because the collagen V-binding domain is relatively small (~19 kDa; see Takagi *et*

al. JBC 1993), it is concluded from the apparent molecular weights of these fragments, which are substantially greater than 19kDa, that additional portions of the thrombospondin molecule must also be present in these fragments (multimers of the 19-kDa region are not a plausible explanation for the higher molecular weights, because the 19-kDa region does not 5 comprise the region of inter-chain disulfide bonds, plus the fact that the gels in Figures 3 and 4 were run under reducing conditions). It is believed that additional portions come from the protease-resistant central core domain of thrombospondin, which can be selected from the group of thrombospondin domains consisting of the region of inter-chain disulfide bonds, the procollagen-like domain, a type 1 repeat, and to some extent a type 2 repeat and a type 3 10 repeat (see Prater CA *et al.* The properdin-like type 1 repeats of human thrombospondin contain a cell attachment site. J Cell Biol. 1991 Mar;112(5):1031-40; Schultz-Cherry S *et al.* The type 1 repeats of thrombospondin 1 activate latent transforming growth factor-beta. J Biol Chem. 1994 Oct 28;269(43):26783-8; Figure 6.2 in Adams JC *et al.* The Thrombospondin Gene Family, 1995, p. 107; and chymotryptic and tryptic fragments of 15 thrombospondin indicated schematically in Figure 1 of this application). See also the sequence ranges given earlier in this Application. Note that several aforementioned peptides, such as, CNSPSPQMNGKPCEGEAR (residues 444-461), RKVTEENKELANELRPP residues 281-297); PQMNGKPCEGEAR (residues 449-461); CEGEAR (residues 456-461; and RKVTEENKE (residues 281-289) are within the protease- 20 resistant central core domain. An antibody against a region outside of a collagen V-binding domain, but present in a thrombospondin fragment present in a cancer patient, is also preferred.

In competition assays, a sample of material (e.g., plasma) that contains thrombospondin fragment(s) and/or thrombospondin is tested for its ability to interfere with 25 the binding of one (or more) of the fragments to a fragment-specific binding agent, preferably an antibody, such as a monoclonal antibody. Under optimal conditions, the ability of the sample to interfere with the binding of the fragment increases monotonically in relation to the amount of similarly binding fragments in the sample. Thrombospondin will also interfere with the binding, but the present inventor has discovered that thrombospondin 30 is present in plasma in significantly smaller amounts than the fragments. In addition, competition assays are easily standardized through the use of known quantities of fragments, synthetic or otherwise, and/or through the use of molecules, such as peptides, that contain an epitope recognized by the binding agent. In one scenario, assay detection is accomplished

through the use of labeled fragments and/or peptides, and addition of a sample that contains a thrombospondin fragment or addition of known quantities of an unlabeled thrombospondin fragment (as a standard) results in competition with the binding of the labeled fragments and/or peptide to the binding agent. Loss of signal upon addition of known quantities of 5 unlabeled or differently labeled thrombospondin fragments is used to standardize the assay.

In addition to an assay of thrombospondin fragments, other examples of platelet activation assays include but are not limited to: a thromboxane assay, a B2 assay, a beta-thromboglobulin (BTG) assay, a platelet-derived growth factor assay, a fibronectin assay, a fibrinogen assay, and a platelet factor 4 assay. Each of these can be assayed by antibody-based assays, such as an ELISA or a competitive ELISA, as is well-known in the art. Platelet activation, including the formation of platelet thrombi, is also indicated by markers that include membrane constituents, such as P selectin (which can be assayed, for example, as soluble P-selectin, which is generated as an alternatively spliced form or is proteolytically released from membrane-bound P-selectin), gpV, and glycocalicin (see Gurney D *et al.*: A 10 reliable plasma marker of platelet activation: Does it exist? *Am J Hematol.* 2002 Jun;70(2):139-44; glycocalicin is the extracellular domain of GP Ibalpha, which can be released from Gp Ib/V/IX complexes on platelets, see Baglia FA *et al.*: Factor XI binding to the platelet glycoprotein Ib-IX-V complex promotes factor XI activation by thrombin. *J Biol Chem.* 2002 Jan 18;277(3):1662-8), as well as platelet microparticles (see Michelson AD & 15 Furman MI: Laboratory markers of platelet activation and their clinical significance. *Curr Opin Hematol.* 1999 Sep;6(5):342-8; Nomura S *et al.*: Relationship between platelet activation and cytokines in systemic inflammatory response syndrome patients with hematological malignancies. *Thromb Res.* 1999 Sep 1;95(5):205-13; Nomura S *et al.*: Function and clinical significance of platelet-derived microparticles. *Int J Hematol.* 2001 20 Dec;74(4):397-404) and certain prostanoids. Assays of these are also well-known in the art. 25

Detection of thrombospondin fragments by Western Blot analysis

The following protocol (Sections I, II, and III) is referred to herein as the "Standard Gel Electrophoresis Protocol" and is preferred for determining whether the size of a 30 fragment is ~ 85 kDa, ~ 50 kDa, ~ 30 kDa or another size. Nevertheless, suitable alternatives for fractionating and detecting molecules and molecular fragments are well-known in the art (see numerous methods articles and texts, as well as protocols from

commercial sources) and are readily applied to the current situation with appropriate modifications.

I. Sample preparation

5 Protease inhibitors added:

1 μ l of leupeptin solution (1mg/ml in sterile water) is added per ml plasma

10 μ l of PMSF solution (1.74 mg /ml in isopropanol) is added per ml plasma

4X sample buffer:

10 dH₂O 4.0 ml / 0.5M tris-HCl 1.0 ml / glycerol 0.8 ml / 10% SDS 1.6 ml / 2-mercaptoethanol 0.4 ml / 0.05 % bromophenol blue 0.2 ml

5 μ l plasma samples are diluted with 20 μ l distilled water, and 25 μ l 2X sample buffer is added, followed by heating (to aid disulfide bond reduction).

15 10 μ l of each sample mixture is then run on the gel.

In an example of an alternative to the Standard Gel Electrophoresis Procedure, to aid reduction and denaturation, blood plasma is mixed with 5% fresh mercaptoethanol and 4-6 M fresh urea and boiled for at least 5 minutes in a fume hood.

20

II. Electrophoresis

Gel electrophoresis is done on SDS-polyacrylamide gels (4% stacking, 10% running gel) in tris/glycine/SDS buffer (see running buffer below, pH 8.3) at 200 V/ 7-8cm at 25°C for 34 minutes. Alternative electrophoretic set-ups and procedures are well-known in the art

25 and can be used (e.g., using gels of about 8%-12% acrylamide; omission of the stacking gel), but should reliably separate 185 kDa, 85 kDa, 50 kDa, and 30 kDa (these are the approximate apparent weights on a reducing gel of thrombospondin and of the three major thrombospondin fragments in plasma). Molecular weight standards were: 184 kDa, 121 kDa, 86 kDa, 67 kDa, 52 kDa, 40 kDa, 28 kDa, and 22 kDa (Figure 3). Other molecular 30 weight markers are suitable as well, but should include markers near to 185 kDa (the approximate weight of thrombospondin on reducing gels) and near to 85, 50, and 30 kDa (the approximate weights on reducing gel of the major thrombospondin fragments present in

plasma). Suitable molecular weight standards are purchasable from a variety of commercial sources, such as Invitrogen Life Technologies (<http://www.invitrogen.com/>).

5X running buffer pH 8.3: Tris Base 15 g / Glycine 72 g / SDS 5 g / distilled water to
5 1 liter

The ~85-kDa thrombospondin fragment electrophoreses close to the 86 kD standard.
The ~50-kDa thrombospondin fragment electrophoreses close to the 52 kD standard.
The ~30-kDa thrombospondin fragment electrophoreses close to the 28-kDa
10 standard.

III. Detection of the fragments on the gels

The fragments may be detected by the Western Blot procedure using antibodies that react with the 85 kDa, 50 kDa, and 30 kDa fragments. TSP Ab-4 antibodies from Lab Vision Corporation (Fremont, CA; <http://www.labvision.com/>) can be used for this purpose (as primary antibody), as can polyclonal anti-TSP antibodies (such as Ab-8, a rabbit polyclonal antibody from Lab Vision). Following standard protocols, proteins from the polyacrylamide gel are transferred to a suitable membrane, unoccupied protein-binding sites of the membrane are then blocked (e.g., by incubation with skim milk), and the membrane is exposed to primary antibody. The presence of TSP Ab-4 antibodies that have bound to thrombospondin or thrombospondin fragments on the membrane can be detected by reacting those antibodies with fluorophore-labeled antibodies against mouse IgG (secondary antibody, *i.e.*, that themselves react with the TSP Ab-4 antibodies), followed by subsequent fluorescence-based scanning of the membrane. Detection of polyclonal anti-TSP antibodies
20 is performed similarly, using appropriate secondary antibodies. Other systems for detection of primary antibody are well-known in the art, including but not limited to other systems for labeling a secondary antibody, such as conjugation to an enzyme, such as horseradish peroxidase. Biotin-avidin systems are also well-known in the art, as are radioactive labeling methods.
25

30

Determination of albumin concentration in plasma samples for purposes of normalizing the Western Blot results.

Gels are run under the same conditions as for the Western Blot, but then stained with

Coomasie Blue. The major band (which is near the 67-kDa standard) is albumin, which is quantified by densitometric scanning.

5 Illustrative, but not restrictive, examples of quantitative assays for TSf (i.e., a thrombospondin fragment or fragments):

Enzyme-linked immunoabsorbant assays (ELISA) and related approaches are well-known in the art (for an example of an ELISA of thrombospondin, but not directed towards thrombospondin fragments, see Tuszyński, G.P., Switalska, H.I., and Knudsen, K.: Modern Methods in Pharmacology in "Methods of Studying Platelet-Secreted Proteins and the 10 Platelet Cytoskeleton," Vol. 4, Alan R. Liss, Inc., New York, p.267-286, 1987). Two types of ELISAs are competitive ELISAs, which require only one anti-TSf antibody, and sandwich ELISAs, which can require two anti-TSf antibodies. Essentially identical assays are also contemplated, in which a binding agent other than an antibody is used.

15 For a competitive ELISA or ELISA-like assay, two sets of wells can be used, one a set of reaction wells and the other a set of pre-mix wells. In the reaction wells, antigen is bound to a surface, such as a plate or a bead (for simplicity, the rest of this description refers to such a surface as a plate or a well, but it is understood that other surfaces can also be used). Here, the antigen would be based on a thrombospondin fragment present in a cancer patient. Said antigen could take a form selected from the group consisting of 20 thrombospondin (TSP) itself, a TSP fragment found in a cancer patient, a TSP fragment that contains a TSP fragment found in a cancer patient, a TSP fragment that is contained within a TSP fragment found in a cancer patient, a peptide that contains an epitope from a TSP fragment in a cancer patient (where said peptide can be synthetic), and a derivatized peptide and/or fragment. The essential requirement for the fragment, protein or peptide coated on 25 the walls is that it can compete with the TSP fragment of interest (for example a fragment in a patient's plasma) for binding to a binding agent, such as an antibody, used in the ELISA. As an illustration, TSP itself can be used, as stated above. TSP can be prepared by activating platelets *in vitro* (which then release TSP-1), followed by purification of this TSP from the platelet-conditioned medium; if standard 96-well microtiter plates are used, 75 ng 30 of TSP-1 in 200 μ L of phosphate-buffered saline can be added per well. Corresponding amounts (molar or mass) of TSP fragments and/or peptides can be used instead, and are preferable, based on ease of preparation and standardization. After binding the antigen to the immobilized surface, additional binding sites on the surface are blocked by standard

protocols (for example, incubation with bovine serum albumin then Tween, both in phosphate-buffered saline).

The pre-mix wells are prepared with no antigen, but then blocked (e.g., with BSA then Tween). These pre-mix wells can be used as convenient reaction vessels for the initial 5 binding of anti-TSf antibody with either known amounts of antigen in solution (for a standard curve) or unknown amounts of antigens in a sample to be tested (see the next two paragraphs).

In order to generate a standard curve, to the pre-mix wells are added different 10 concentrations of a standard antigen in solution. The standard antigen might (as described elsewhere herein) be selected so as to quantify the amount of thrombospondin fragments of the invention, the amount of a subset of thrombospondin fragment or fragments, the amount 15 of thrombospondin, or their combined total. The antigen may be synthetic, isolated from a cancer patient, isolated from an individual without cancer, or isolated from any other appropriate source, including but not limited to recombinant material. As indicated above, 20 the immobilized antigen in the reaction wells and the antigen in solution in the pre-mix wells do not have to be the same, but they should both react with – and thereby eventually compete for – the binding agent (such as a primary antibody) used in the assay. As an 25 illustrative example, if TSP-1 itself is the standard antigen in solution in the pre-mix wells, 0, 2, 5, 10, 20, 40, 60, and 80 ng can be added per well, in PBS-Tween, in volume of 110 uL per microtiter well. Corresponding amounts (molar or mass) of TSP fragments or peptides 30 can be used instead, and are preferable, based on their ease of preparation and standardization. These wells will be used to generate a standard curve.

Unknowns (i.e., samples in which it is desired to quantify the concentration of a TSP 25 fragment) are also added, to separate pre-mix wells. For plasma samples, it is typical to dilute them beforehand, say, with PBS-Tween. This can be important, to bring the amount of TSf down into the range of the standard curve, and also to dilute potentially interfering 30 substances in plasma (one such interfering substance may be fibrinogen, which can bind TSP and some TSP fragments). Total volume should be the same as for the soluble antigen standards. Diluted binding agent, such as an antibody (e.g., in 110 uL), that reacts against a TSP fragment found in a cancer patient is then added. Note that the antigen immobilized in the reaction wells and the antigen in solution in the pre-mix wells must be chosen to also react against this binding agent. An incubation is performed, to allow antigen-antibody binding (or target-binding agent binding) to occur in the pre-mix wells.

An aliquot (e.g., 200 μ L) of liquid from each premix well (standards and unknowns) is then transferred to an antigen-coated reaction well, followed by an incubation (as a blank, some wells can receive buffer only, such as PBS-Tween). After this incubation, liquid is removed from the antigen-coated reaction wells, and the wells are washed. If a primary 5 antibody is used as the binding agent, enzyme-conjugated secondary antibody (specific against the primary antibody) is then added to the wells, followed by an incubation to allow it to bind to whatever primary antibody has bound to the immobilized TSf on the plate. This step is followed by detection (for example, if the secondary antibody is conjugated to alkaline phosphatase, detection can be accomplished with Sigma phosphatase substrate 10 followed by absorbance readings at 405 nm). A standard curve is plotted, and quantities of a TSf in the unknown samples are calculated based on the standard curve. Note that higher amounts of TSf in the sample will result in less primary antibody bound to the immobilized antigen on the well, and hence less signal from the secondary antibody. Similar detection methods are used if the binding agent is a non-antibody.

15 Sandwich ELISAs and ELISA-like assays are also contemplated. In this case, a first anti-TSf antibody (or a first non-antibody binding agent that binds TSf) is immobilized on a plate, a bead, or another surface, and it is used to capture the TSf in a standard or unknown sample. The first antibody is often polyclonal, but this is not a requirement. Detection of 20 captured material is then accomplished with a second anti-TSf antibody. The second antibody is often monoclonal, but this is not a requirement. As is well-known in the art, the first and second antibodies should not substantially interfere with each other's access to the captured material. Detection can be accomplished with an enzyme-linked antibody specific to the second anti-TSf antibody. Again, if the first (capturing) binding agent and/or the second (detecting) binding agent is a non-antibody, similar methods are used.

25 Many variants well-known in the art are contemplated for these competitive and sandwich ELISAs and ELISA-like assays. For example, non-enzymatic methods, such as radioactive, fluorescent, biotin-avidin-based methods, and other methods to detect the anti-TSf antibody are contemplated. Also, automated assays, such as ones performed on a clinical autoanalyzer, are contemplated. Also, various anti-TSf antibodies are contemplated, 30 including but not limited to polyclonal antibodies, monoclonal antibodies, anti-peptide antibodies, antibodies against a TSP fragment present in a cancer patient, antibodies against a TSP fragment generated *in vitro*, and antibodies against a TSP fragment generated *in vitro* by proteolysis. Single-chain antibodies are also contemplated, as are non-antibodies.

For the sandwich ELISA, one option is the use of color-coded microbeads (microspheres) with immobilized anti-TSf antibody to capture, then a fluorescent second anti-TSf antibody to detect. The detection apparatus reads each bead, one at a time, assaying for bead color as well as the signal from the second anti-TSf antibody. The advantage here

5 is that several different analytes can be assayed at once, such as one group of beads for full-length TSP (or an epitope outside of what circulates in substantial concentration in a cancer patient) and another group of beads, of a different color, for a TSP fragment. Or, one group of beads to assay an epitope present in the ~85-kDa TSP fragment that is not present in the ~50- or ~30-kDa fragments, and another group of beads to assay an epitope present in the

10 ~50-kDa fragment but not the ~30-kDa fragment (this is followed by a numerical calculation to yield the amounts of ~85-kDa fragment and of ~50-kDa fragment separately). An example of this use of color-coded beads can be found at
<http://www.lincoresearch.com/lincoplex/technology.htm>, the web site for Linco Research, Inc.

15 Another option for analyzing multiple analytes is SearchLight™ Proteome Arrays, which are multiplexed sandwich ELISAs, currently adapted for the quantitative measurement of two to 16 proteins per well. It is understood herein that the method can also be used for protein fragments, such as one or more thrombospondin fragments. Using a spotting technique, 2 to 16 target-specific antibodies are bound to each well of a microplate,

20 although it is understood that this number can be expanded with improved spotting techniques and/or larger wells. Following a standard sandwich ELISA procedure, luminescent signals are imaged with a cooled CCD (charged coupled device) camera. The image is then analyzed using Array Vision™ software. The amount of signal generated at each spot is related to the amount of target protein in the original standard or sample. Values

25 for specific proteins and/or protein fragments can be calculated based on the position of the spots and comparison of density values for unknowns to density values for known standards (and TSP fragments or peptides can be used as standards). The SearchLight™ technology is available through Pierce Boston Technology Center (<http://www.searchlightonline.com/>), including customized arrays using proprietary reagents from outside Pierce or assay targets

30 not currently available at Pierce (see http://www.searchlightonline.com/custom_array.cfm).

Other assay methods are also contemplated. They include but are not limited to immunoblotting, dot-blotting and immunoturbidimetric assays (for a detailed example of this

last approach with another plasma protein, see Levine, D.M. and Williams, K.J.: Automated measurement of mouse apolipoprotein B: convenient screening tool for mouse models of atherosclerosis. *Clin. Chem.* 43:669-674, 1997), as well as blotting and/or turbidimetric assays that use binding agents in general. Other competitive assays are also contemplated, 5 such as ones in which material in a standard and an unknown competes with one or more labeled peptides, one or more labeled TSP fragments, and/or labeled TSP for binding to an agent that binds TSf, such as an anti-TSf antibody (the label is then used for detection and hence quantification). One example of this approach is to immobilize an anti-TSf antibody, and then add sample mixed with labeled peptide, labeled TSP fragments, or labeled TSP, so 10 that sample and labeled material compete for binding to the immobilized antibody (note that this approach requires only one anti-TSf antibody). Binding of labeled material is then quantified. It is understood that any of these assays, including immune-based and non-immune-based assays, can be automated. It is also understood that potentially interfering substances in unknown samples can be diluted, removed, inhibited, avoided (for example, in 15 the case of fibrinogen, by using epitopes away from a fibrinogen-binding region of TSP), and/or compensated for.

Use of thrombospondin fragments as immunogens to generate fragment-specific antibodies:

A purified preparation of fragments (e.g., by elution of fragments from the gel, by 20 immunoprecipitation or antibody column or other immune-based purification methods, by recombinant DNA techniques, by chemical synthesis, or by a combination of synthesis and/or purification methods) is injected into a rabbit or rabbits with any of the standard adjuvants used with peptide immunogens and antibodies are collected using protocols well known in the art. For small peptides, linkage to a carrier, such as keyhole limpet 25 hemocyanin or bovine serum albumin, is well-known in the art. Injection into other animals is also well-known, including but not limited to a goat, sheep, chicken, turkey, donkey, dog, cat, rat, and mouse. Monoclonal antibodies can be prepared using antibody-producing cells obtained from any immunized animal, but the technology is most easily available for the mouse (for example, antibody-producing cells from an immunized animal are fused with an 30 immortal cell, then clones of fused cells are screened for their production of antibody against one or more thrombospondin fragments of interest).

It is understood that the methods disclosed herein are readily applied to other members of the thrombospondin gene family, including but not limited to TSP-2 (for a description of the thrombospondin gene family, see The Thrombospondin Gene Family by JC Adams, RP Tucker, & J Lawler, Springer-Verlag: New York, 1995; de Fraipont F et al. 5 *Trends Mol. Med.*, 7:401-407, 2001; and elsewhere). It is also understood that the methods disclosed herein are readily applied to other animal species of economic and/or emotional importance, including but not limited to pets, animals used in breeding, racehorses, and racing dogs.

10

EXAMPLES

Western Blot analysis of plasma samples from cancer patients

Electrophoresis was done according to the Standard Gel Electrophoresis Protocol described above.

15

Table I shows plasma and serum samples obtained for analysis.

Sample	Plasma/Serum	Cancer	Stage/Grade	Age/Sex	Comment
A	plasma	colon T2	I/G2	57/F	Ascending
B	plasma	colon T3	II/G2	71/M	Ascending
C	plasma	prostate	II/Gleason 6	71/M	DRE-abnormal
D	plasma	prostate	II/Gleason 5	63/M	DRE-abnormal
E	plasma	lung T2	IB/G2	67/M	Squamous
F	serum				TSP is released from platelets during clotting, and proteases are activated during clotting.
G	plasma	no cancer	N/A	F	lichen planus, a non-cancerous inflammatory disorder

Table 1

The results are shown in Figures 2 and 3, and the quantitative data are summarized in Table 2.

Approx MW (kDa)	A Colon-1	B Colon-2	C Prostate-1	D Prostate-2	E Lung	F Serum	G No cancer
85	0.572	0.847	1.175	1.292	1.142	1.434	0.526
	108.8%	161.1%	223.6%	245.7%	217.4%	272.9%	100.0%
50	0.534	0.666	1.037	1.416	1.809	2.722	0.596
	89.7%	111.8%	174.0%	237.7%	303.6%	456.9%	100.0%
30	1.210	1.401	1.687	1.593	1.988	7.351	1.424
	85.0%	98.4%	118.5%	111.9%	139.6%	516.3%	100.0%
Total Ab4 signal	2.316	2.914	3.898	4.301	4.939	11.507	2.545
	91.0%	114.5%	153.2%	169.0%	194.1%	452.1%	100.0%
Albumin signal above bkg	24020	26723	25187	27073	23888	4359	26110

5 **Table 2: Quantitation of thrombospondin fragments, normalized for sample loading**
Numbers refer to the strengths of TSf signal from the Western blot (Figure 3), normalized to the albumin signal from Coomassie staining (Figure 2 and final row of numbers in this Table). Percentages indicate the ratio to the no-cancer sample (sample G).

10

The results summarized in Table 2 represent data generated by densitometric scanning of the photographic film generated by fluorescent staining of the TSP Ab-4 Western Blot (See Figure 3). Thus, for very dark signals, such as the band or group of bands around 30 kDa, the fact that the signals on film saturate when very strong means that 15 increases compared to the no-cancer control sample are seriously under-estimated. This is not particularly evident in the serum sample, which served as the positive control for increased signal, owing to platelet activation (much less serum was loaded, as is evident from the albumin signal; so even though it generated a strong normalized signal, it did not saturate the film nearly as much).

20 To obtain the data for Table 2, the signal (above background) for the Western Blot was determined and that signal was normalized to the albumin signal (above background) for the gel shown in Figure 2. Table 2 shows the normalized signal (e.g., 0.572) with the

percentage (e.g., 108.8%) underneath the normalized signal being the percentage of the "no-cancer" signal.

The molecular weight standards used were 184 kDa, 121 kDa, 86 kDa, 67 kDa, 52 kDa, 40 kDa, 28 kDa, and 22 kDa. Based on the given molecular weights and the relative 5 positions of the standard bands versus the TSP Ab-4 bands and groups of bands, it was concluded that the TSP Ab-4 signals were in three general bands or groups of bands, at approximately 85, 50, and 30 kDa (see Figure 3). Notice, for example, the relative strength of signals at around 185 kDa (thrombospondin) *versus* around 85, 50, and 30 kDa (fragments). It is clear that there is overwhelmingly more signal from the fragments than 10 from thrombospondin itself. Thus, detection of specific fragments, as disclosed in the current inventions, is preferred over detection of the TSP molecule itself, or general detection of epitopes that occur throughout the whole TSP molecule, or detection of epitopes outside of those contained within the specific fragments demonstrated herein.

The plasma samples from cancer patients (lanes A-E) came from Golden West 15 Biologicals, Inc. of Temecula, CA. The serum sample (lane F) was from a non-cancerous individual. The no-cancer control plasma (lane G) came from an individual with lichen planus, a non-cancerous but inflammatory skin condition.

The serum sample (Lane F) was prepared by deliberately clotting the blood. Protease 20 inhibitors were not added to sample F until after clotting had been completed and the serum had been harvested. Ideally for the current invention, however, blood is not allowed to clot during sample collection (activation of platelets during clotting causes release of thrombospondin, which was used here on purpose to increase the signal from sample F), and protease inhibitors are added promptly during sample collection (not done for sample F because the clotting cascade involves activation of proteases).

25 The predominance of thrombospondin fragments, as opposed to thrombospondin itself, in sample F is consistent with a) platelet activation and release of thrombospondin, plus b) activation of proteases of the clotting cascade, which evidently cleaved the newly released thrombospondin.

Plasma samples from Golden West Biologicals were samples from individuals with 30 relatively early disease. The first colon cancer sample (lane A) was from an individual with stage I, grade G2 disease. All other cancer samples (lanes B-E) came from individuals with stage II disease (except for lane E, which was stage IB). Plasma from patients with such relatively early stage cancers would be expected to have a lower concentration of

thrombospondin fragments than plasma from patients with more advanced cancers. Nevertheless, the robustness of the technique is demonstrated by the fact that (1) increased levels were found with the earlier stage cancers, and (2) gel scanning was done under conditions in which portions of the detecting film were saturated or nearly saturated.

5 All cancer samples show an increased signal from the 85-kDa band (or group of similarly electrophoresing bands). All but the stage I sample show increased signal from the 50-kDa band (or group of bands), as well as increased total Ab-4 signal. All but the two early colon cancer samples show increased signal from the 30-kDa band (or group of bands). Thus, the detection and quantitation of specific thrombospondin fragments works to
10 distinguish even relatively early cancer patients from a no-cancer control who has a non-cancerous disease. These thrombospondin fragments are well-suited for diagnostic assays because (a) they have specific molecular weights (or molecular weight ranges); and (b) they contain specific epitopes. The present results provide validation for other fragment-based approaches, including (but not limited to) non-competitive ELISA and ELISA-like assays,
15 and competition assays.

Figure 4 shows the results of an independent gel analysis of the samples. The samples were denatured then run on a 12% gel, transblotted, and then stained with the same TSP Ab-4 that we used before. Unlike the blot shown in Figure 3, the denaturation step here included urea, and detection used an enzymatic colorometric method that is based on
20 horseradish peroxidase conjugates and the BioRad Opti-4CN substrate kit (see <http://www.discover.bio-rad.com/>), not fluorescence as before. Along the left edge of lane 1, there are from top to bottom, the following handwritten numbers evident: 1, 97, 66, 45, 30, 20, and 14, respectively. With the exception of 1, the numbers correspond to the positions where standard proteins of corresponding molecular weights (in kDa) had
25 electrophoresed.

In Figure 4, Lanes 2 through 6 correspond to patients A though E, respectively, in Table 1. Lanes 1 and 7 through 9 show the electrophoresis patterns of thrombospondin. The results confirm that:

- a) there is virtually no TSP in the plasma samples (the first plasma lane shows some
30 TSP at its appropriate monomer molecular weight, but this is certainly spill-over from the vastly overloaded first sample lane);
- b) there are indeed TSP fragments in the plasma samples; and

c) the fragments have molecular weights of about 28, 50, and a faint band around 90 kDa. In this blot, the TSf bands are very sharp, implying well-defined molecular weight fragments (presumably a purely technical improvement, owing to better denaturation in the presence of urea). As in Figure 3, there are a number of less prominent fragment bands at 5 other molecular weights. It is understood that a thrombospondin fragment in any of these bands can also be assayed and used in diagnosis and screening and in kits.